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Title: Endothelin-1 mediated vasoconstriction and cerebral hypoperfusion in Alzheimer's disease
Endothelin-1 mediated vasoconstriction and cerebral hypoperfusion in Alzheimer’s disease

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Abstract

Cerebral hypoperfusion, associated with cognitive decline, is a hallmark of vascular dementia and is now recognised to be a key pathological feature in the early stages of Alzheimer’s disease (AD). This thesis aims to explore the hypothesis that pericyte-mediated contraction, as a result of elevated endothelin-1 (EDN1; a potent vasoconstrictor), is a major contributor to cerebral hypoperfusion in AD. Electrical impedance assays (xCELLigence) have been used to characterise and then explore the impact of amyloid-beta (Aβ) peptides on EDN1-mediated contraction of human brain-derived vascular pericytes in vitro. The expression of endothelin receptors type-A (EDNRA) and type-B (EDNRB) have been characterised in cultured pericytes after 1- and 24-hour Aβ exposure and in human post-mortem brain tissue in AD. The major findings indicate that human brain-derived vascular pericytes contract in response to EDN1 acting via EDNRA; adult brain-derived pericytes are less responsive than foetal pericytes to EDN1; EDN1-mediated pericyte contraction is dysregulated in the presence of physiological concentrations of Aβ peptides and that exposure to Aβ induces the gene and protein expression of EDNRA (or increased ratio of EDNRA:EDNRB). In human brain tissue, EDNRA level (but not gene expression) is upregulated in AD and was associated with Aβ plaque load. The findings presented in this thesis support the hypothesis that increased pericyte contraction (because of elevated EDN1), and dysregulated pericyte contraction (because of increased Aβ), contribute to the impairment of neurovascular coupling in AD. In conclusion, abnormal EDN1-evoked pericyte contraction is likely to be a major contributor to capillary vasoconstriction and thus cerebral hypoperfusion in AD.
Publications

Acknowledgements

I am very grateful to the MRC GW4 DTP for funding my PhD scholarship, for without their generous aid, this project would not be possible.

My sincerest thanks go to my supervisor’s Dr Scott Miners and Prof. Seth Love for their unfailing support and teachings throughout the project, without whom I would have not been able to complete the PhD.

I would also like to thank all the other members of the Dementia Research Group for their help in the lab and for sharing their expertise. Some special words of gratitude go to my fellow ‘Lab Legends’ Kelly and Rob who have taken the greater part of this PhD journey with me. Thank you for your support, advice and help in the lab and equally ensuring my time here was as fun as possible.

Finally, I want to say a huge thank you to my family, friends and my partner Ralph for their unwavering love and encouragement every step of the way.
COVID-19 Statement

There has been a significant, unavoidable portion of time spent away from all research facilities and resources due to the closure of campus facilities related to the COVID-19 pandemic. This impacted both data collection and analyses and had knock-on effects, leading to consumables expiring and subsequent delays in resupply (as most are sourced overseas). Multiple periods of self-isolation due to exposure to and contraction of COVID-19 also significantly impacted my research. Firstly, as these experiments were terminated prematurely and then on return, I needed to re-establish the cell cultures, these unanticipated interruptions have significantly impacted and prolonged the time required for experiments and data collection and reduced the time I would have otherwise spent progressing other aspects of research. Secondly, in line with social distancing, the number of personnel and time spent in the labs since the first lockdown has been reduced – laboratory staff have been working on a booking/rota system. On many occasions, due to high demands for workspace, I have not been able to come to work as the maximum working capacity was reached.

I regularly met and discussed the progress of my PhD with my supervisors throughout the pandemic. Together, we have on numerous occasions adapted and revised our plans to attempt to mitigate the impact of COVID-19. We replaced more risky animal experiments with more adaptable/ flexible experiments using human post-mortem brain tissue that is routinely employed in all the labs here. We also adjusted the scope of the project to focus on gene expression and quantitative fluorescence and combining the results of these with pre-existing biochemical measurements.

The tissue request for human post-mortem brain tissue was, however, further delayed due to an already existing backlog of requests. Staff redundancies that arose from the pandemic led to staff shortages which meant the additional tissue cases could not be cut and distributed on time. Cell culture work was further disrupted by additional periods of self-isolation from contraction of and close contact with someone who had COVID-19. This led to further discard of live cell cultures. Orders for laboratory consumables had been substantially delayed, which significantly affected the proposed PCR work in particular. This was due to prioritised delivery of PCR consumables to government testing facilities.
Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University’s Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate’s own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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<tr>
<td>~</td>
<td>Approximately</td>
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<tr>
<td>&lt;</td>
<td>Less than</td>
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<tr>
<td>α-SMA</td>
<td>Alpha-smooth muscle actin</td>
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<tr>
<td>ACh</td>
<td>Acetyl-choline</td>
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<td>AChE</td>
<td>Acetyl-cholinesterase</td>
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<td>AChEI</td>
<td>Acetyl-cholinesterase inhibitor</td>
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<td>AChR</td>
<td>Acetyl-choline receptor</td>
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<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
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<tr>
<td>ADE</td>
<td>Aβ degrading enzyme</td>
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<tr>
<td>ADRDA</td>
<td>Alzheimer's Disease and Related Disorders Association</td>
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<td>aHBVP</td>
<td>Adult human brain vascular pericytes</td>
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<td>ApoE</td>
<td>Apolipoprotein-E</td>
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<td>Apolipoprotein-E gene</td>
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<td>BBB</td>
<td>Blood brain barrier</td>
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<td>CAA</td>
<td>Cerebral amyloid angiopathy</td>
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<td>CADASIL</td>
<td>Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy</td>
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<td>DLB</td>
<td>Dementia with Lewy Body</td>
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<tr>
<td>eNFT</td>
<td>Extra neuronal neurofibrillary tangles</td>
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<td>Early-onset Alzheimer’s disease</td>
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<td>FAD</td>
<td>Familial Alzheimer’s disease</td>
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<td>FFPE</td>
<td>Formalin-fixed paraffin embedded</td>
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<td>fHBVP</td>
<td>Foetal human brain vascular pericytes</td>
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<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
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<tr>
<td>g</td>
<td>Grams</td>
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<td>Intramural periarterial drainage</td>
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<tr>
<td>I</td>
<td>Litre</td>
</tr>
<tr>
<td>LOAD</td>
<td>Late-onset Alzheimer’s disease</td>
</tr>
<tr>
<td>LRP1</td>
<td>Lipoprotein receptor-related protein 1</td>
</tr>
<tr>
<td>m</td>
<td>Milli</td>
</tr>
<tr>
<td>M</td>
<td>Molar concentration</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin-associated glycoprotein</td>
</tr>
<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
</tr>
<tr>
<td>MMP9</td>
<td>Metalloproteinase 9</td>
</tr>
<tr>
<td>MMSE</td>
<td>Mini Mental State Examination</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>n</td>
<td>Nano</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangles</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NIA</td>
<td>National Institute of Aging</td>
</tr>
<tr>
<td>NICE</td>
<td>National Institute for Health and Clinical Excellence</td>
</tr>
<tr>
<td>NINCDS</td>
<td>National Institute of Neurological and Communicative Disorders and Stroke</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>NVU</td>
<td>Neurovascular unit</td>
</tr>
<tr>
<td>p</td>
<td>Pico</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF-B</td>
<td>Platelet-derived growth factor-B</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>Platelet-derived growth factor-B receptor</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PLP1</td>
<td>Proteolipid protein-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------</td>
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</tr>
<tr>
<td>PSEN-1</td>
<td>Presenilin-1</td>
</tr>
<tr>
<td>PSEN-1</td>
<td>Presenilin-1 gene</td>
</tr>
<tr>
<td>PSEN-2</td>
<td>Presenilin-2</td>
</tr>
<tr>
<td>PSEN-2</td>
<td>Presenilin-2 gene</td>
</tr>
<tr>
<td>P-tau</td>
<td>Phosphorylated tau</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end products</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPL13</td>
<td>60S ribosomal protein L13</td>
</tr>
<tr>
<td>RPL13</td>
<td>60S ribosomal protein L13 gene</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>sPDGFRβ</td>
<td>Soluble platelet-derived growth factor-β</td>
</tr>
<tr>
<td>SVD</td>
<td>Small vessel disease</td>
</tr>
<tr>
<td>T-tau</td>
<td>Total tau</td>
</tr>
<tr>
<td>UBE2D2</td>
<td>Ubiquitin-conjugating enzyme E2 D2</td>
</tr>
<tr>
<td>UBE2D2</td>
<td>Ubiquitin-conjugating enzyme E2 D2 gene</td>
</tr>
<tr>
<td>VaD</td>
<td>Vascular dementia</td>
</tr>
<tr>
<td>VCI</td>
<td>Vascular cognitive impairment</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>WMH</td>
<td>White matter hyperintensities</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>μ</td>
<td>Micro</td>
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Chapter 1  Introduction

1.1  Dementia

Dementia is the most common neurological syndrome amongst the aged(1). It is a condition that affects learning, memory, and behaviour. It can be severely debilitating, affecting a person’s ability to carry out daily tasks. Dementia is not only a huge burden on the individual but also on the person’s family and friends who, often, become the person’s primary source of care. In December 2017, the World Health Organisation reported an estimated 50 million people worldwide were living with dementia, a number that is predicted to triple by 2050(2). Dementia not only poses great social challenges but imparts a huge financial burden costing the U.K. an estimated £23.6 billion in 2014, which is projected to rise to approximately £59.4 billion by 2050(3).

Dementia is an umbrella term, used to encompass multiple cognitive disorders. The three main causes of dementia are Alzheimer’s disease (AD) which makes up 60% of dementia cases(4), Vascular Dementia (VaD) accounting for up to 20%(5), and Dementia with Lewy Bodies (DLB) making up 15% of total dementia cases(6). Other less common causes, accounting for less than 5% of dementias, include frontotemporal dementia, posterior cortical atrophy, primary progressive aphasia, and other much rarer forms of dementia such as Creutzfeldt-Jakob disease(7). The proportion of each dementia type is illustrated in Figure 1.

![Pie chart showing the percentage of different dementia types. Alzheimer’s disease (60%); Vascular dementia (20%); Dementia with Lewy Body (15%) and other rarer forms of dementia (< 5%).](image-url)
1.2 Alzheimer’s disease

1.2.1 Background

The most common cause of dementia is AD, accounting for between 60-80% of cases of dementia globally (8). AD was first described by Alois Alzheimer in 1906 (9) upon an autopsy of a 55-year-old woman named Auguste Deter. Deter had died from a disorder that affected her cognition and behaviour, displaying signs of paranoia, confusion, disturbed sleep and aggression (10). During the autopsy, Alzheimer noted two distinct pathologies in the brain: plaques and neurofibrillary tangles (NFTs). These characteristic neuropathological hallmarks are still used today to confirm a diagnosis of AD.

There are two main types of AD: early-onset AD (EOAD) and late-onset AD (LOAD). EOAD accounts for between 1-5% of all cases. It occurs before the age of 65 years and has a more aggressive disease course. It includes familial AD (FAD), which results from inheritance of a genetic autosomal dominant mutation that predisposes individuals to onset of disease at a younger age (<65 years). LOAD, or sporadic AD, is the most common form of the disease, it accounts for between 95-99% of all cases, and is associated with the onset of dementia in people aged 65 or older. The pathophysiology of LOAD involves complex interactions between genetic risk factors and environmental and lifestyle factors (11).

AD is typically characterised by a deterioration in cognitive ability, an impairment in learning and memory, as well as in speech and language. Other behavioural changes that are associated with AD include aggression, confusion, depression, changes in sleep pattern and changes in appetite (12, 13).

Population-based studies from 1990 until 2008 have reported an estimated median survival time of ~3-5 years (14) following the onset of clinical symptoms in AD. However, variation in the initial diagnosis of AD makes estimating disease duration difficult. Prospective studies starting with disease-free patients provide the most impartial estimates for survival rates, however, few have been carried out in recent years and those that are available are limited by sample size (15, 16).

1.2.2 Diagnosis of AD

The first set of widely adopted criteria for clinically diagnosing AD was set out in 1984 by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer’s Disease and Related Disorders Association (ADRDA) (17). The NINCDS-ADRDA
criteria, as they are commonly referred to, were widely used for their high sensitivity (81%) and specificity (70%) (18). A definitive diagnosis then (and still today) requires post-mortem neuropathological examination. Post-mortem diagnosis requires the presence of NFTs and neuritic plaques (19); cases of dementia without these must be classified as non-AD (20). The NINCDS-ADRDA guidelines have since been revised to include criteria that allow distinction between AD and associated “spectrum disorders” including mild cognitive impairment (MCI) and prodromal AD (21). Nowadays, a clinical diagnosis of AD is made following an assessment of patient history, imaging, and cerebral spinal fluid (CSF) biomarker analysis, as well as cognitive testing. One such cognitive test is the Mini Mental State Examination (MMSE) which has proven useful in helping to diagnose moderate to severe AD (with an approximate sensitivity of 80-90%) but is less sensitive to milder cases of the disease (22).

Imaging modalities are also useful in improving the diagnostic accuracy of AD. Magnetic resonance imaging (MRI) can be used to assess structural changes in the brain. The most consistent and clinically relevant MRI abnormality in AD patients is the appearance of tissue atrophy in the medial temporal lobe (including the hippocampus and amygdala). The atrophy then spreads to the parietal and frontal lobes, and leads to dilation of the lateral ventricles and reduction in total brain volume (23). Positron emission tomography (PET) is also used to detect amyloid-β (Aβ) plaque load in vivo. One of the advantages of Aβ PET is that Aβ plaque burden can predict progression of MCI to AD (24) however, Aβ burden poorly correlates with disease progression and severity in established AD (25). Recent advances have allowed for PET tau imaging, enabling localisation of tau pathology in the brain. Tau imaging is useful for monitoring disease progression and staging. One recent study characterising the relationship between PET tau imaging and Aβ CSF measurements found that the former performed better than PET Aβ in predicting AD (25). Other imaging modalities such as computed tomography (CT) are still widely used to evaluate large cerebral lesions. CT scans are not as powerful compared to MRI scans at detecting medial temporal atrophy but are nonetheless diagnostically useful.

Profiling CSF biomarkers, obtained from a lumbar puncture, are useful in supporting a diagnosis of AD. The operation is low cost compared to imaging modalities and if performed correctly has a low complication rate and high diagnostic yield, but some discomfort is experienced (26). CSF biomarkers, indicative of AD, show abnormal concentrations in pre-clinical stages of the disease, allowing for early AD diagnosis before symptom onset (27). CSF biomarkers are preferred over blood and/or plasma biochemical markers because the CSF is in direct contact with the brain interstitial fluid and are more accurate for diagnosis of AD. CSF has an unrestricted bi-directional
flow and the levels of the relevant markers are not influenced by the periphery due to segregation by the CSF-blood barrier(28). The three clinical biomarkers examined in the CSF are related to three main pathological changes that occur in AD: Aβ peptide deposition, tau accumulation and neuronal loss. The Aβ peptide composed of 42 amino acids (Aβ1-42) is produced by dysfunctional cleavage of the amyloid precursor protein (APP) (discussed in further detail in section 1.2.5). Its low solubility and tendency to aggregate into extracellular plaques is reflected in a decrease in concentration in the CSF in those with AD. Tau proteins, present in the cytosol of neurons where they help stabilise the microtubules, are hyperphosphorylated due to an imbalance of kinase and phosphatase activity. Phosphorylated tau at threonine (p-tau181) detaches from the microtubules and is released from apoptotic cells and this forms NFTs and is thus detected as an increase in the CSF. Neuronal injury results in the release of total tau (t-tau), increasing the t-tau concentration in the CSF(29). The combined use of all three has a high diagnostic power to discriminate between those with AD and those who are healthy, with a sensitivity of 92% and a specificity of 89%(30). Recently discovered novel CSF and plasma p-tau biomarkers, phosphorylated at threonine-217 and threonine-231, along with p-tau-181 have been found to be significantly elevated in pre-clinical AD when minimal Aβ pathology is detectable(31, 32).

Evidence suggests changes in these biochemical and imaging AD biomarkers do not all happen at once but occur sequentially. For these biomarkers to be useful for disease staging, the order of this sequence must be fully understood. In an attempt to conceptualise this, Jack et al. created an AD biomarker model showing the temporal relationship between the five most common biomarkers during disease progression. The model predicted Aβ plaque biomarkers were the earliest features in the disease as measured by CSF Aβ1-42 and amyloid by PET. Second were biomarkers of neuronal injury including tau-mediated dysfunction. Third were MRI biomarkers including changes in brain structure preceding onset of clinical symptoms including deteriorating memory and clinical function(33). This model has been updated with the collection of more extensive data from longitudinal studies. For example, one study that followed over 100 MCI patients over approximately 9 years after a baseline CSF analysis, showed that CSF Aβ1-42 was abnormally reduced in patients who converted to AD during follow-up(34). Additionally, CSF p-tau and t-tau were abnormally increased at baseline in those with MCI who went on to develop AD(34). The original model has since been revised to reflect recent developments in our understanding of the temporal relationship between AD biomarkers and disease stage (see section 1.3.2).
1.2.3 **Treatment of AD**

There are several medicines currently licenced by either the National Institute for Health and Clinical Excellence (NICE) and the National Institute of Aging (NIA) that can help treat symptoms and slow the progression of AD. Acetylcholinesterase inhibitors (AChEIs) such as galantamine, donepezil and rivastigmine work by inhibiting acetyl-cholinesterase (AchE), the enzyme responsible for the breakdown of acetylcholine (Ach), a neurotransmitter which is important in learning and memory(35). AchEIs are commonly prescribed to treat mild to moderate AD. The evidence that AchEIs alter the progression of the disease is controversial. In a study investigating the effects of donepezil hydrochloride in AD patients, those receiving the drug showed significantly reduced rates of hippocampal tissue atrophy compared to control patients(36). However, there is evidence from *in vitro* work that suggests AchEIs promote Aβ deposition(37). This is also supported by an *in vivo* study showing accelerated AD pathology (at 6-months rather than 9-months) in transgenic mice expressing human APP when crossed with mice expressing human AchE(38). The cognitive effects of AchEIs have shown to be improved water-maze acquisition and memory retention in aged Fisher rats compared to controls(39). In humans, AchEIs are associated with improved memory as demonstrated by improved MMSE score and a lower mortality risk(40).

For treating more severe AD, an N-methyl-D-aspartate type receptor (NMDAR) antagonist, memantine, can be given primarily to manage cognition but has been shown to slow Aβ production(41-43). NMDAR plays a crucial role in the synaptic transmission and plasticity thought to underlie learning and memory(44). Perturbed NMDAR activation and downstream Ca²⁺ signalling has been implicated in AD and correlates clinically with progressive decline in memory(45, 46). A meta-analysis of 12 trials looking at the effects of memantine on cognitive test scores demonstrated that treatment with memantine improved MMSE scores(47). In June 2021, the U.S Food and Drug Administration approved the first potentially disease-modifying therapy. Aducanumab (Aduhelm) is an Aβ-directed monoclonal antibody, which preferentially binds to aggregated forms of Aβ, reducing the number of plaques in the brain and slowing disease progression. The trial results showed that participants who had been given aducanumab for up to three years experienced a time- and dose-dependent reduction in plaque level as well as a reduction in cognitive decline as tested by MMSE. Currently the Food and Drug Administration require a further phase 4 confirmatory trial to verify the clinical benefit as some controversy exists around its efficacy. The European agency did not grant a licence for its clinical
use due to insufficient evidence of a clinically significant benefit to patients and the fact that its testing was limited to patients with MCI or early-AD only(48).

1.2.4 Neuropathology of AD

Post-mortem assessment of AD brains show the most consistent macroscopic changes are cortical tissue atrophy, enlargement of the lateral ventricles(13), widening of the sulci and lower brain weight(49) (Figure 2A). These are a result of a decrease in cortical tissue reflecting a loss of neurons, dendrites, and degeneration of nerve fibres(50, 51). In late-stage disease, there is significant neuronal loss from the hippocampus and entorhinal region of the brain(52).

The two microscopic hallmarks observed in AD, that were first described by Alois Alzheimer, are Aβ plaques and NFTs. Both hallmarks can be seen in non-diseased brains, however, marked increases in their abundance, specifically in regions affected by disease, are typically seen in AD(13) (Figure 2B-C).

Figure 2. Neuropathological hallmarks of AD

(A) Macroscopic changes seen in the AD brain including tissue atrophy and enlargement of the ventricles (left), compared to healthy control brain (right). (B) Aβ plaques and (C) neurofibrillary tau tangles immunolabelled in human post-mortem brain tissue sections indicated by arrows. 20X magnification. Images courtesy of the South West Dementia Brain Bank, University of Bristol.
The Aβ peptide is the main constituent of plaques in the AD brain. Aβ is generated from the sequential enzymatic cleavage of APP (see section 1.2.5 for more detail). Two types of Aβ-plaques exist: neuritic and diffuse plaques. Neuritic plaques normally comprise abnormal, dystrophic neurites surrounding a core of Aβ and can be anywhere between 20 and 25 µm in diameter. Diffuse plaques are much larger, having a diameter of several hundred microns. Diffuse plaques do not have an Aβ core or surrounding dystrophic neurites.(53).

Ultrastructural studies show that NFTs are composed of paired helical filaments of hyper-phosphorylated tau approximately 10 nm in diameter. Tau’s normal function is to stabilise the microtubules of the cytoskeleton within the cell but is found to be hyperphosphorylated and aberrantly misfolded in AD, rendering it dysfunctional.(54). Three distinct NFT morphologies have been identified; the first is pre-NFT or ‘diffuse’ NFT, defined within the cytoplasm of otherwise normal-looking neurons. The second is mature or fibrillar intraneuronal NFT (iNFT) which contain filamentous aggregates of tau in the cytoplasm which displaces the nucleus to the periphery of the cell. The third and final morphology is extra-neuronal ‘ghost’ NFT (eNFT) which are apparent after the death of NFT-containing neurons and are easily identified as they do not contain a nucleus(55, 56).

1.2.5 Aβ processing

The two main forms of Aβ - Aβ1-40 and Aβ1-42 are derived from enzymatic processing of APP (although smaller amounts of other fragments including Aβ1-43 and Aβ1-48 are also produced). APP is a single-pass transmembrane protein with a large extracellular domain and is produced predominantly in neurons(57). Its exact physiological function is unknown although some studies indicate it has a neuroprotective role and supports neuronal growth and repair. Evidence in support of this comes from in vivo studies that have shown enlarged neurons in mice overexpressing APP(58). Mouse models with the deletion of APP show little or no phenotypic effect, suggesting that a loss in APP is minimally detrimental to the adult mouse(57).

APP is subject to two processing pathways (illustrated in Figure 3); the amyloidogenic and non-amyloidogenic pathway(57). Both pathways involve sequential cleavage of APP by two enzymes. In the amyloidogenic pathway, APP is first cleaved by β-secretase (BACE-1), a transmembrane aspartic protease. In the non-amyloidogenic pathway, APP is first cleaved by α-secretase. Both enzymatic cleavages remove almost the entire ectodomain of APP. This leaves either a β- or α-C terminal membrane-bound fragment. These fragments are then cleaved by γ-secretase, a multiunit complex made up of presenilin-1 (PSEN1), presenilin-2 (PSEN2), nicastrin, Aph1 and
Pen-2. In the amyloidogenic pathway, cleavage at +40 or +42 amino acids releases Aβ1–40 or Aβ1–42. In the non-amyloidogenic pathway, cleavage of APP releases a p3 peptide - a small amyloid fragment, the function of which is currently unknown(59).

The majority of Aβ produced is Aβ1–40 that accounts for approximately 90% of total Aβ. About 5-15% is Aβ1–42(60) which is more hydrophobic and has a greater tendency to aggregate and deposit within the brain parenchyma(61). Although Aβ1–40 is more soluble it can deposit and can accumulate in the walls of large blood vessels in a process known as cerebral amyloid angiopathy (CAA)(62).

Figure 3. APP processing pathways
Sequential cleavage of APP by two enzymatic processing pathways; (A) the non-amyloidogenic which involves enzymatic cleavage of APP by α-secretase and then by γ-secretase to produce p3 peptide. (B) The amyloidogenic pathway involves sequential cleavage by β-secretase (BACE-1) and then by γ-secretase to produce Aβ. Figure adapted from O’Brien. R, Wong. P, 2011.

1.2.6 Aβ aggregation

Aβ is produced as a monomer which self-aggregates into dimers and trimers. Further oligomerisation produces protofibrils and then fibres (Figure 4). This process is termed fibrillogenesis and is based on nucleation-dependent polymerisation. These fibres undergo a conformational change that promotes aggregation into large extracellular plaques(63). Amyloid deposits are approximately 6–10 nm and arranged in crossed β-pleated sheets where the polypeptide chain is perpendicular to the fibril axis and parallel hydrogen bonding(64). Smaller monomeric and dimeric forms of amyloid are soluble whereas larger oligomeric forms of
amyloid are insoluble and are neurotoxic in vitro(64). Soluble Aβ makes up most Aβ in the healthy brain (50% and 23% for Aβ1-40 and Aβ1-42 respectively) but a small proportion in the AD brain (2.7% and 0.7%)(65). This progressive shift (because of a change in soluble as well as increase in insoluble) of Aβ1-40 and Aβ1-42 from soluble to insoluble pools in AD, with a marked increase in insoluble Aβ1-42, suggest a mechanistic role of insoluble Aβ in AD progression and development.

![Diagram showing aggregation of Aβ](image)

Figure 4. Aggregation of Aβ

Aβ monomers self-aggregate to form dimer and trimers. These larger soluble amyloid species self-aggregate into protofibrils which undergo a conformational change to form insoluble β-pleated sheet structures. These insoluble fibres aggregate and deposit as extracellular plaques.

How Aβ mediates neurotoxicity is still under debate. Evidence suggests a likely mechanism is Aβ-mediated formation of pores or channels within the cell membrane leading to disrupted Ca$^{2+}$ homeostasis(66) and oxidative stress(67). Arispe et al. demonstrated that insertion of Aβ oligomers into the cell membrane formed cation-specific channels(68) and others have shown cytosolic calcium increases as a result(69). Conversely, some evidence suggests that Aβ oligomers do not exhibit pore-forming properties themselves, but change the conductive properties of the lipid bilayer, disrupting ion flow and leading to cellular dysfunction(70).

Extracellular Aβ oligomers may also cause neurotoxicity via binding to the cell surface and leading to functional disruption of several receptors. Several possible mechanisms are currently under investigation. Recent works by Yamamoto and colleagues suggested that Aβ oligomers induce nerve growth factor (NGF) receptor-mediated death of neurons(71). NGF can cause cell death through activation of the p75 neurotrophin receptor, a member of the tumour necrosis factor family of receptors. Supporting evidence comes from another study that showed Aβ-
derived ligands may alter NGF-mediated signalling(72). Other reports on receptor-mediated toxicity by Aβ oligomers have shown disrupted NMDAR-mediated long-term potentiation, specifically of downstream signalling pathways including cAMP response element-binding protein(73). Studies have also demonstrated the ability of Aβ oligomers to mimic NMDAR blockade by reducing NMDAR activation affecting NMDAR-specific Ca²⁺ signalling that is vital for cell viability(74). One study identified the cellular prion protein (PrP⁰) as an Aβ oligomer-binding receptor(75). Binding to PrP⁰ leads to disrupted interaction and subsequent communication between co-receptors like NMDAR.

Another potential mechanism under investigation is intracellular Aβ oligomer accumulation within neuronal cells(76, 77). Intraneuronal Aβ accumulation has been demonstrated in cultured cells, rodent models as well as AD patients(78-80) however, it remains to be determined whether this intracellular accumulation is a result of secreted Aβ being taken back up by the cell. How these intracellular pools of Aβ cause neurotoxicity is unclear; however, some studies allude to these oligomers inhibiting proteasome and deubiquitinating enzyme activity that are important in intracellular trafficking of receptors thus leading to cellular dysfunction(81). In support of this, another study demonstrated that inhibited proteasome activity in transgenic mouse models of AD cause cellular dysfunction through disrupted clearance of tau(82).

1.2.7 Aβ clearance

Whilst aberrant production of Aβ through dysfunctional APP processing is a widely accepted mechanism for Aβ accumulation in familial AD, impaired removal of Aβ has recently come under investigation and is suggested to be important in promoting Aβ accumulation, particularly in sporadic AD.

Clearance of Aβ can be either enzymatic or non-enzymatic. Aβ-degrading enzymes (ADEs) that have a role in mediating Aβ clearance include mainly proteases such as neprilysin and insulin-degrading enzyme(83). Most of the ADEs exhibit endopeptidase activity, cleaving peptide bonds between amino acid within Aβ while others including matrix metallopeptidase-9 (MMP9) and glutamate carboxypeptidase-II cleave at the carboxyl end of the Aβ peptide sequence(83). Non-enzymatic clearance of Aβ includes interstitial fluid drainage into the CSF, removal by the phagocytic activity of microglia and receptor-mediated clearance across the blood-brain barrier (BBB)(83).
Waste removal from the CNS is essential for maintaining normal brain function. The glial-lymphatic or ‘glymphatic’ system, is a fluid clearance system recently identified in the rodent brain(84). It utilises aquaporin 4 (AQP4) water channels on glial cell end-feet to promote the removal of fluids from the brain to the interstitial fluid and out of the brain(85). Glymphatic dysfunction has been demonstrated in animal AD models, most likely associated with altered AQP4 expression. Disrupted glymphatic function in AQP4-knockout mice exhibited reduced Aβ clearance compared to control mice(85). Deletion of AQP4 did not alter the expression of Aβ protein, nor any proteins involved in its production or degradation, suggesting deletion of AQP4 affected only clearance(86). While the cause for perturbed AQP4 expression in AD is unclear, studies have alluded to the fact that sleep may have bearing on the functionality of the glymphatic system. Clearance of metabolic products occurs mainly at night during sleep and disturbed sleep is a known risk factor for AD. This is supported by a study showing glymphatic drainage of infused Aβ doubled during sleep compared to awake states(87) and others reporting marked reductions in clearance in sleep-deprived mice(87, 88). Collectively these studies suggest that sleep is important in functional glymphatic drainage of Aβ.

Aβ is also cleared from the brain via the intramural periarterial drainage (IPAD) pathway. Extensive work has been done to study the transport of solutes along the IPAD pathway during aging(89) and under various disease conditions such as CAA and ischemic stroke(90). Collectively, these works demonstrated that soluble tracers injected into the brain interstitium reach the basement membrane of cerebral arteries and that their distribution and deposition closely mimics the distribution of Aβ in CAA(91). Together, these data indicate soluble Aβ drains across the basement membrane of cerebral capillaries and surrounding concentric layers of smooth muscle cells (SMC) of arteries(92, 93). Failure of IPAD could explain the vascular deposition of Aβ in CAA.

CAA is commonly seen in the AD brain where Aβ deposits in the walls of cerebral arteries and capillaries. This results from failed removal of Aβ via interstitial drainage and has been suggested to promote Aβ accumulation(94). The causes of failed drainage are unclear, however, some studies have proposed that Aβ binds to collagen within the vessel wall(95) as a result of dysfunctional transport molecules such as apolipoprotein-E (ApoE)(96). It also could be a result of a perfusion deficit in the drainage pathways caused by mechanisms unknown at present. Microglia are the brain’s resident phagocytes, found to be highly associated with Aβ plaques(97, 98). These cells can degrade and remove Aβ via phagocytosis of Aβ plaques(99). Their precise
role in the brain during AD is still hotly debated because opposing roles have been observed. In the early stages of AD, microglia are neuroprotective, promoting Aβ clearance through phagocytosis(98). However, aged microglia in later stages of the disease demonstrate an age-dependent decrease in phagocytic activity against Aβ, with one study observing a loss of phagocytic activity at 6 months in mice(100). Overactivated microglia also cluster around Aβ plaques and contribute to a neuroinflammatory environment that is believed to be a major contributor in the pathophysiology of AD. Others have observed two different types of microglia that exist in the brain, resident and bone marrow-derived. Those derived from the bone marrow have been described as neuroprotective, as supported by studies in transgenic mouse models that highlight the microglia’s participation in the removal of Aβ plaques by phagocytosis and cytokine release(101).

Receptor-mediated transport of Aβ is by far the most studied model of Aβ clearance. Low-density lipoprotein receptor-related protein 1 (LRP1) is a scavenger receptor, first found to play a role in cholesterol transport and metabolism. It is one of the most characterised scavenger receptors in mediating Aβ clearance. Receptor-mediated transport and removal of Aβ across the BBB is both concentration- and age-dependent, meaning that Aβ clearance is faster in younger animals and at lower Aβ concentrations(102). LRP1 is also expressed in neurons, microglia, pericytes and astrocytes. Chaperone proteins including ApoE and alpha-2-macroglobulin can bind to Aβ and help promote its uptake via LRP1(103). ApoE-ε2 promotes efficient uptake and removal of Aβ whereas ApoE-ε4 show diminished rates of degradation(104). Perhaps it is for this reason, that ApoE-ε4 is considered the more pathogenic variant of APOE and is why ε4 carriers have a higher risk of dementia.

Increased Aβ deposition as a result of augmented production and impaired clearance has given rise to the widely accepted amyloid cascade hypothesis(105) (Figure 5). It is known that exposure of neuronal cell cultures to Aβ results in cell death(106, 107), suggesting a potential role of Aβ in disease pathogenesis. Aβ accumulation has been shown to initiate downstream pathways, i.e., act as a trigger, and induce tau phosphorylation that ultimately results in synaptic remodelling and neuronal death. Aβ oligomers can also mediate direct neurotoxicity(108). Plaque load is often saturated before the onset of clinical symptoms and hence does not correlate with cognition (as would be expected if Aβ plaques were the sole pathogenic entity in AD(109, 110)). In vivo models that overexpress Aβ show deficits in learning, memory and cognition before plaque formation and deposition(111) suggesting that other mechanisms underlie cognitive deficits seen in AD.
People with FAD (fewer than 5% of all cases) carry an autosomal dominant genetic mutation that predisposes them to AD. The genetic mutations occur in one of three genes. The first is in the gene that encodes APP on Chromosome 21. A total of 39 mutations have been found in APP which all affect APP proteolysis in favour of over-producing $\text{A}\beta_{1-42}$ (112). The second and third mutations are in PSEN-1 and PSEN-2. These genes encode the proteins presenilin-1 (PSEN-1) and PSEN-2, respectively, which are proteins that form part of the $\gamma$-secretase complex that is involved in APP processing (Figure 3). Mutations in these, particularly in PSEN-1, are the most common mutations in FAD. The mechanisms by which mutations lead to FAD are still largely unknown but appear to centre around an overproduction of $\text{A}\beta_{1-42}$ or change the ratio of $\text{A}\beta_{1-42}$: $\text{A}\beta_{1-40}$. Two hypotheses exist; the first is the amyloid hypothesis which proposes that mutations that facilitate the amyloidogenic processing and cleavage of APP resulting in an increased ratio of $\text{A}\beta_{1-42}$ to $\text{A}\beta_{1-40}$, either through an increased $\text{A}\beta_{1-42}$ production or decreased $\text{A}\beta_{1-40}$ production, or a combination of the two (113). However, $\text{A}\beta$ accumulation is not a requisite for AD.
feature of neurodegeneration because some dementias, like frontotemporal dementia lack Aβ pathology. Thus, Aβ deposition is a poor correlate with dementia pattern and severity, whereas synaptic loss correlates well with clinical symptoms of AD(114). Thus, the presenilin hypothesis was posited to provide an alternative explanation of neurodegeneration in AD independent of Aβ. This hypothesis suggests that a reduction in functional PSEN-1 leads to impaired brain function. This is drawn from in vivo work that has shown PSEN-1 to be important in learning and memory as well as in neuronal growth(115) via cleavage and processing of functional Notch receptors that facilitate neuronal cell growth and survival(116).

Sporadic AD or LOAD usually includes those who develop AD after the age of 65 and is the most common form of AD accounting for between 95-99% of cases. The heritability of LOAD has been estimated to be as high as 79%(117). The APOE gene encoding ApoE, a polymorphic glycoprotein found in the brain and liver as well as in macrophages and other cells(112), is the biggest genetic risk factor for sporadic AD. Its primary role in the brain is to regulate cholesterol and transport of lipids to aid in neuronal growth and repair. Three major allelic variants at a single gene locus exist; ε2, ε3 and ε4, which have a worldwide occurrence of 8.4%, 77.9% and 13.7% respectively. The prevalence of the ε4 allele increases to approximately 40% in those with AD(118). The differing presence of cysteine vs. arginine amino acids at sites 112 and 158 of ApoE affects its binding of lipids and receptors. ε3, the most common isoform, has cysteine at position 112 and arginine at 158. ε2 has two cysteines at these positions and ApoE-ε4 has two arginines. ApoE-ε4 has greater binding affinity to cholesterol and other lipids than ApoE-ε3 and ApoE-ε2, which has a major effect on ApoE’s functions. Those possessing the ε2 variant have been found to have a reduced risk of developing LOAD, whereas ε4 carriers have an increased risk(118). As well as increasing a person’s risk of developing AD, the ApoE-ε4 allele has also been associated with worsened disease pathology and clinical function. ApoE-ε4 is associated with increased amyloid deposition(119), impaired Aβ clearance, and accelerated memory decline and hippocampal atrophy(120). The association of increased amyloid and ApoE-ε4 allele is also seen in animal models of Aβ accumulation(121).

Since the first large genome-wide association study (GWAS) in 2009(122), many gene loci have since been identified that are associated with the risk of AD(123). Functional analyses including gene-, cell- and tissue-specific analyses have identified immune cells and microglia as cells of interest, containing genes that were most significantly associated with disease risk(124). A recent study using blood vessel isolation and nuclei extraction for sequencing has shown up to 30 of the top 45 GWAS genes previously identified are expressed in the vascular system in
humans including NOTCH3 (VSMC function and survival), COL4A2 (vascular basement membrane protein) and KCNE4 (potassium voltage gated channels), revealing an important role of the vascular system in AD(125). These differentially expressed genes were implicated in vasoconstriction (actin-mediated cell contraction) and blood flow dysregulation(125) and resembled those found in other diseases that have marked impaired blood flow, like CADASIL.

1.2.9 Modifiable risk factors and dementia

Growing evidence from epidemiological studies, modelled by the 2017 Lancet Commission on dementia prevention, intervention, and care, have identified nine potentially modifiable risk factors for dementia: smoking, obesity, depression, hypertension, low social contact, less education, hearing impairment, diabetes, and physical inactivity. Three additional risk factors: high alcohol intake, air pollution and traumatic brain injury have also been recently recognised(126).

Higher childhood education levels are associated with a reduced risk of developing dementia(127-129) however, cognitive ability plateaus in late adolescence suggesting cognitive stimulation in early life is crucial. A large study in China, however, reported that cognitive stimulation later in life (in people over 65 years) was also associated with reduced risk of dementia incidence(130).

Increasing evidence supports a Mediterranean diet as effective in reducing the risk of developing dementia(131, 132). A Mediterranean diet typically consists of a low intake of saturated fats, a low to moderate intake of dairy, a high intake of vegetables and unsaturated fats and a low to moderate intake of alcohol(133). Close adherence to a Mediterranean diet is associated with protection against cognitive decline, specifically memory(134). High adherence to such a diet was associated with a 1.5-3.5-year protection against the development of AD based on a 3-year brain-imaging study(135). Those eating a Mediterranean diet have also been shown to have longer telomere length and higher telomerase activity, important for cellular proliferation and tissue regeneration. Additionally, reduced oxidative stress was also observed(133). Conversely, a high-fat diet is associated with increased risk of obesity and type-2 diabetes which both promote the development of cognitive dysfunction and most probably dementia. In a triple transgenic AD mouse model, a high-fat diet for 4 months, caused marked memory impairment(136) but no difference in levels of Aβ or tauopathy suggesting a high-fat diet leads to cognitive deficits independent of amyloid. However, some recent studies have
found no association whatsoever between diet and risk of dementia(133), suggesting that adherence to a healthy diet may not be effective at reducing the risk of cognitive decline or AD.

AD and cerebrovascular disease share risk factors including diabetes, hypertension, and hypercholesterolemia. The presence of cerebrovascular disease also lowers the threshold for dementia(137) and can accelerate the conversion of MCI to AD(138).

There is a strong association between smoking and the risk of developing AD. Smoking has been shown to lead to cognitive impairment, more specifically verbal memory impairment(139) and cognitive decline is directly proportional to the number of cigarette packs smoked per day(140). Historically, smoking was prescribed to improve cognitive deficits as nicotine has been shown to improve short-term memory and inhibit amyloidosis(141). However, more recent studies have demonstrated that smoking does increase the risk of developing AD, reporting a significant increase in relative risk of AD development of 1.45 (95% CI, 1.16-1.80) (142-144). Studies in animal models have also shown that exposure to nicotine leads to an increase in tau phosphorylation(145) as well as amyloid deposition(146). The underlying mechanism behind how smoking may increase an individual’s risk of developing AD is still unclear and further studies are needed to identify potential mechanisms. A caveat to this and many other epidemiological studies and meta-analyses is that the diagnosis of AD has been based on a clinical diagnosis, and we know that clinical diagnosis of AD is often inaccurate and ignores most co-contributors to dementia.

Hypertension is an established risk factor for VaD involving atherosclerosis and arteriosclerosis pathogenesis(147). There have also been numerous large-scale studies highlighting the association between hypertension and AD. In the Honolulu Aging Study, increased midlife systolic blood pressure was associated with poorer cognitive performance 25 years later(148). In this study, the risk for an intermediate and poor cognitive function was increased by 7% and 9% respectively for every 10-mmHg increase in systolic blood pressure. A 15-year longitudinal study by Skoog and colleagues reported findings consistent with that of the Honolulu Aging Study. Participants who developed dementia had both increased systolic and diastolic blood pressure 10-15 years prior to the onset of clinical symptoms(149). Other works also show a positive correlation between increased systolic blood pressure and the development of AD after 27 years(150). Others have reported that a drop in blood pressure precedes cognitive decline and subsequent development of dementia(151, 152), while others have reported no association whatsoever(153). The disparity between these findings is unclear. In the aim to understand the pathogenic mechanism behind this association, work in animal models have demonstrated
increased BBB permeability, hypoperfusion and neuroinflammation in adult mice models of hypertension. These were followed by an increased deposition of Aβ as early as 4 weeks after(154). Human studies have similarly shown increased Aβ burden in those with midlife hypertension. A post-mortem study found that midlife hypertension was associated with CAA, Aβ plaque formation and NFTs(155). This work has been supported by PET-imaging of Aβ showing increased burden is associated with midlife hypertension(156). Collectively, these works suggest midlife hypertension may lead to cerebrovascular injury, subsequent BBB dysfunction, impaired clearance and Aβ accumulation. Conversely, a meta-analysis conducted by Guan and colleagues found no association between hypertension and AD and in a separate study, the authors found no effect of antihypertensive medication on risk of AD development(157). This was supported by further meta-analyses conducted by Power et al. who found no significant association between systolic or diastolic hypertension and AD(158).

Exercise can improve vascular health and has been shown to reduce the risk of developing VaD and AD(159). Individuals who have had regular exercise throughout life on average have larger brain volumes and improved executive cognitive function(160). A 1-year intervention of aerobic exercise in older adults was found to increase hippocampal volume, an area severely affected by AD, by approximately 2%(161). As well as disease prevention, physical activity also has a positive effect on improving symptoms. AD patients carrying out regular exercise showed a slower decline in their capability to perform basic everyday tasks(162), as well as improved memory and cognitive function(163). While these data support a beneficial effect of physical activity on clinical symptoms, the effects on disease pathology in AD are limited in clinical studies owing to the difficulties of studying the effects in humans. Transgenic rats expressing human APP, placed on a treadmill to exercise for 30 minutes every day for 1 month, showed a decline in amyloidogenesis and tauopathy as well as neuroprotection(164). Similar transgenic animal studies show physical activity leads to a reduction in Aβ deposition, larger hippocampal volume, and reversal of behavioural symptoms(165, 166). Acute physical activity also increases brain-derived neurotrophic factor (BDNF) production. BDNF is a mediator in neurogenesis and plays an essential role in the formation of memories(133). BDNF also shifts APP processing in favour of the non-amyloidogenic pathway (see section 1.2.5) by activating α-secretase and, therefore, reducing the levels of toxic Aβ peptide(167). However, other studies have shown mixed results with one reporting no association between exercise and improved cognition(168) so this relationship is still controversial. The fact that AD and cerebrovascular diseases share common risk factors suggests that they share common pathogenic mechanisms.
1.3 Vascular contribution to dementia

Reduced cerebral blood flow (CBF), or cerebral hypoperfusion, and resulting brain tissue ischaemia, are the defining pathological processes in VaD, which accounts for between 10-20% of all dementia cases. Vascular dysfunction, including reduced CBF and BBB breakdown, is also a major and largely underestimated contributor to cognitive decline and disease progression in AD(169). There is evidence of cerebrovascular damage, including CAA(170) and small vessel disease (SVD)(171), in most AD cases.

1.3.1 Cerebrovascular disease and Alzheimer’s disease

Cerebrovascular changes are often seen concurrently with AD and correlate with cognitive decline(172-174). Post-mortem studies reveal SVD in the majority of people with dementia; ischaemic white matter damage has been found in approximately 60% of AD patients(175). Lines of evidence in support of this include one study which, by using the US National Alzheimer’s Coordinating Centre database to identify over 5000 patients with dementia, found that of those who had only AD (no evidence of any other neurodegenerative disease), 80% had vascular pathologies including SVD and CAA(173).

CAA can cause vessel occlusion, ischemia and can lead to the eventual death of neurons and accelerate AD. CAA has been reported in most AD cases(170) and has been shown to propagate over time in AD mouse models(176). In a Honolulu-Asia Aging Study, compared to non-demented non-CAA patients, individuals with both CAA and AD had greater cognitive impairment than those with CAA or AD alone(177, 178). CAA is also known to increase the risk of several vascular pathologies including ischaemic strokes, haemorrhages, and white matter lesions that all contribute to a decline in cognition(179).

Cerebral SVD, including white matter hyperintensities (WMH), microbleeds in the brain and tissue infarcts, is suggested to contribute to AD development. One study showed a significantly increased risk of AD in those with WMH 8 years prior to the onset of clinical symptoms(180). In support of this, another study found parietal WMH was a predictor of AD and was significantly correlated with the existence of tau(181). Numerous cross-sectional studies have been carried out to investigate the association between WMH and Aβ. An investigation in an AD and MCI cohort revealed a significant association between WMH and plasma Aβ1-40 as well as an association between MWH volume and Aβ1-42(182). Consistently, other studies report associations between WMH and plasma Aβ(183) and CSF Aβ levels in MCI and AD(184). Results from one study by Rosano et al. indicated a marked association between subclinical infarcts and
AD progression(185). Cerebral microbleeds do not appear to be as strongly associated with the risk of AD-specific dementia. In one longitudinal study, Miwa and colleagues reported cerebral microbleeds showed an increased risk of dementia but no significant association with AD-specific dementia(186). Consistently, another study showed that microbleeds were associated with a risk of AD of the same magnitude of that of non-AD dementia(187). Concerning AD-specific pathology, numerous studies have shown a significant association between cerebral microbleeds and Aβ, all showing that CSF-Aβ1-42 was lower in those with MCI and AD(188-190).

1.3.2 Disease modelling

Work by Iturria-Medina and colleagues, using human disease modelling, indicates vascular dysfunction is the most-likely initial pathological event in the development of LOAD(191). A spatiotemporal multifactorial causal model that combines different neuroimaging modalities and cognitive measurements, was used to predict spatiotemporal alterations in the brain such as Aβ load, vascular flow, and cognitive integrity, among others. A key feature identified by this model was peripheral vascular and inflammatory alterations. The authors observed abnormal protein measurements in both the plasma and CSF, in particular interferon-γ-induced protein 10 (IP-10)(191). Altered plasma IP-10 concentrations reflect peripheral vascular inflammation, a characteristic feature of ageing and related neurodegenerative diseases(192). Altered BBB permeability might be associated with abnormal insulin resistance which in turn may alter the regulation and transport of small molecules across the BBB as well as lead to changes in brain glucose, Aβ and tau levels(193). Another key feature identified by this model was reduced CBF. Using arterial spin labelling-MRI (ASL-MRI) to measure CBF, the model identifies reduced CBF as one of the earliest changes in LOAD(191, 194) (Figure 6).
Cerebral hypoperfusion

Reduced CBF (i.e., cerebral hypoperfusion), is an early feature of human AD (191, 194) and in mice overexpressing APP (195) as well as humans expressing the ApoE-ε4 allelic variant (196, 197). As of yet, no direct causal relationship has been demonstrated between reduced CBF and AD, however, it has been shown to cause ischaemic tissue damage in AD which might underpin and accelerate disease pathogenesis in AD. A reduction in CBF occurs before the development of AD.
of AD(198) and in vivo work has shown vascular abnormalities occur well before any observable behavioural or pathological changes associated with AD(199).

1.4.1 Cerebral hypoperfusion and cognition

Whether this perfusion deficit is global or restricted to specific areas of the brain has been of interest. Some studies have shown associations between age-dependent reductions in global cerebral perfusion and attention deficit(200) as well as in grey matter in AD compared to healthy controls(201). However, most studies suggest a regional perfusion deficit in AD that spreads with the progression of the disease. ASL-MRI studies demonstrate this most notably in the parietal cortex, bilateral posterior cingulate gyrus and precuneus in subjects with MCI and AD compared to those with normal perfusion in healthy brains(202-205). The earliest perfusion deficit is seen in the medial parietal cortex or precuneus(206) and spreads throughout the cortex mirroring the regional spread of Aβ pathology(207) as supported by imaging studies(208). Cerebral hypoperfusion can predict cognitive decline. Using transcranial Doppler to measure CBF velocity, a study has observed that individuals with diminished CBF velocity are associated with an increased risk of developing dementia 6.5 years later(198).

1.4.2 Cerebral hypoperfusion and Aβ

Induced ischaemia in animal models and in vitro modelling of ischaemia by culturing cells in conditions of oxygen-glucose deprivation, results in increased Aβ production(209, 210) due to increased amyloidogenic processing of APP and impaired Aβ clearance. Serum Aβ_{1-42} has been shown to increase significantly in man following transient global cerebral ischaemia due to cardiac arrest(211) or after traumatic brain injury(212, 213). CAA and arteriosclerotic SVD, both of which reduce CBF, have been demonstrated to reduce Aβ clearance from the brain(213) which may contribute to the increased Aβ deposition in the brain during the disease. In support of this, a study using PET imaging to measure Aβ concluded that with age, an increase in deposition is apparent and that arterial stiffness is strongly associated with progressive Aβ accumulation in the brain(214). Conversely, one study has reported no association between reduced CBF and Aβ deposition compared to in areas with normal perfusion(215). This could be attributed to the small sample size and undefined area of perfusion deficit measured.

Aβ and cerebral hypoperfusion have been described as having a bidirectional relationship due to recent findings that show that the Aβ peptide can cause reduced CBF. This is not only through the development of CAA but through inducing vasoconstriction. Work in animal models has shown Aβ peptides reduce CBF by directly acting on cerebral arteries(216) via binding to RAGE
receptors on endothelial cells and SMCs(217), and by disrupting homeostatic mechanisms and cerebral autoregulation of the vasculature(199).

1.5 Cerebral vasculature

The brain accounts for only 2% body mass but receives 20% of cardiac output and consumes approximately 20% of all available oxygen for normal function(218); it cannot store energy, making strict regulation of blood flow and oxygen delivery to the brain vital for survival. A highly developed network of blood vessels exists to carry oxygen throughout the body and to the brain.

Blood supply to the brain (Figure 7) is received via two major routes, the internal carotid arteries, and the vertebral arteries. Anterior and posterior blood vessel circuits are interconnected by communicating arteries, together making up the Circle of Willis at the base of the brain. The brainstem and midbrain are mainly supplied by the posterior blood vessels whereas the cerebral hemispheres receive blood by both anterior and posterior routes. A network of smaller cerebral blood vessels and capillaries exist that maintain the BBB which helps regulate the exchange of solutes between the brain and blood and protect the brain from an influx of blood-derived neurotoxic agents(219).

Figure 7. Anatomy of the Circle of Willis

An imbalance in oxygen homeostasis can lead to tissue hypoxia and eventual cell death. To help combat this, there are mechanisms in place that help to ensure sufficient blood supply. Cerebral autoregulation is the ability of the brain to maintain relatively constant overall blood flow despite fluctuations in mean arterial blood pressure(220). Autoregulation occurs throughout the body but is particularly important in the brain, likely due to the high demand of oxygen and constant blood supply. A second mechanism the brain employs is a process by which increases in blood flow are matched to the metabolic need of the tissue and areas where neuronal activity, glucose and oxygen utilisation are increased(221). This is known as functional hyperaemia, or neurovascular coupling, and is particularly important in the brain due to the rapid local fluctuations that occur in neuronal activity and hence metabolic demand.

1.5.1 Capillary architecture

Capillaries are composed of vascular endothelial cells, connected via adherens and tight junctions, covered by a basement membrane(222). The endothelial cell vessel organisation can take three different phenotypes: continuous, discontinuous, and fenestrated. Continuous endothelium is mainly found in the brain. With its undisrupted basement membrane and tight junctions connecting endothelial cells, it makes up the BBB ensuring tight control of fluid and molecule exchange. A discontinuous endothelium is predominantly found where unrestricted cell trafficking is important such as in the spleen and liver. Intercellular gaps in fenestrated endothelium are found in the kidneys and intestine where fluid exchange is important(223).

A collection of different cell types within capillaries works together to regulate blood flow and control supply of oxygenated blood to activated brain regions, a process known as neurovascular coupling(224). Together, these cells make up what is known as the neurovascular unit (NVU) (Figure 8). The cellular composition of the NVU along the vasculature varies at different locations. Generally, the NVU comprises endothelial cells, astrocytes, neurons, and mural cells including vascular smooth muscle cells (VSMC) and pericytes(225, 226). Neurons are considered the pacemaker of the NVU. They detect small changes in their nutrient and oxygen supply and relay this to adjacent astrocytes or interneurons(227) to influence the vascular tone and consequent blood supply. Astrocytes are a key cell in the NVU as they can detect changes in glutamate levels in neurons and convert these changes into vasomodulatory signals to the rest of the NVU(228, 229). Astrocytes can communicate simultaneously with both neurons and blood vessels and are extremely versatile in their ability to form connections with multiple cells via gap junctions. Such connections allow interplay between not only neurons but also pericytes, VSMC and capillaries themselves through physical contact and communicating chemically via...
gliotransmitter release(230). VSMC are mainly found at the arteriolar end, pericytes are predominantly localised around the capillary and what is described as stellate VSMC appear to localise at the venular end(231). Signals from neurons and astrocytes ultimately activate these mural cells to elicit vasomodulation, reduce or increase vascular resistance and consequently increase or decrease CBF.

Figure 8. Cells of the NVU
Simplified illustration of the NVU at the capillary, comprising neurons (blue), astrocytes (yellow), endothelial cells (red) and pericytes (brown). Image adapted from Zlokovic et al. 2016.

1.6 Pericytes
Pericytes were first described by Eberth in 1871, however, the discovery of pericytes is commonly assigned to Charles-Marie Rouget, a French scientist who in 1873 first reported a population of contractile cells surrounding endothelial cells in small blood vessels(232). It was Zimmermann in 1923 who first coined the term “pericyte” and during the 30 years that followed, numerous publications surfaced describing these cells, some of them questioning their contractility [reviewed elsewhere(233)]. These inconsistencies were attributed to different experimental techniques but are most likely because of the heterogeneous nature of these cells.
1.6.1 Morphology

Pericyte density varies between different organs and vasculature beds. They are highly expressed in the brain at a ratio of 2:1 endothelial: pericytes compared to the skeletal musculature where the endothelial: pericyte ratio is 10:1 (234). Pericytes alongside VSMC are mural cells and are primarily found surrounding capillaries (235). They are heterogeneous in morphology; some appear as umbrella-like structures embedded within the basement membrane of the endothelium (236) but are also found in the walls of some small arterioles and venules (235). Pericytes can also have long projections that extend around the endothelium. Primary cytoplasmic processes extend along the abluminal surface of the endothelium and usually span across several endothelial cells. At capillary branch points, pericyte processes typically span along both branches in a Y-shape (Figure 9A). Smaller secondary processes extend, usually perpendicularly, from the primary processes that wrap around the vessel tube (232). Pericyte processes can adopt three distinct forms: one that wraps around the vessel at a more defined position covering a smaller surface area with finger-like projections (Figure 9B); one that forms around the vessel with continuous cell extensions covering a large portion of the vessel (Figure 9C); and one that elongates along the vessel lengthways, reflecting migrating pericytes following vessel injury (Figure 9D) (236).

![Figure 9. Different morphologies and distribution of pericytes along the vasculature](image)

Illustration depicts the different morphologies and distribution of pericytes along the capillary. (A) Pericytes at capillary branch points with the characteristic ‘Y’ shaped morphology with two processes running along each branch with secondary smaller processes perpendicular. Pericyte processes can adopt 3 distinct forms: (B) finger-like projections covering a defined location, (C) continuous cell extensions around the vessel and (D) those that traverse along the vessel lengthways.
1.6.2 Identification

There is no single pericyte-specific marker; however, several markers when used in combination with morphology can help identify pericytes from other cell populations. Perhaps the pericyte marker most commonly used is platelet-derived growth factor receptor-β (PDGFRβ). It is a tyrosine kinase receptor involved in the PDGF-B/PDGFRβ signalling axis between endothelial cells and pericytes and helps mediate pericyte recruitment during angiogenesis (the formation of new blood vessels)(237). Chondroitin sulphate proteoglycan 4 is an integral membrane proteoglycan in pericytes and is involved in pericyte recruitment to tumour blood vessels(238). Contractile proteins of the cytoskeleton, α-smooth muscle actin (α-SMA)(239) and desmin(240) are also useful markers found in pericytes. It is important to note that whilst used to identify pericytes, these markers are also found on other non-pericyte cell populations. For example, PDGFRβ is found on SMCs and some neurons and α-SMA and desmin are expressed in SMCs(232). Also, expression of these markers can vary, being upregulated, or downregulated in different developmental and disease stages which can make identification and isolation difficult.

A combination of morphology and anatomical distribution (i.e., presence of positively labelled cells on blood vessels that are less than 10 µm in diameter) has traditionally been used to identify and distinguish pericyte populations.

Other molecular signatures of pericytes are distinct from VSMC. Transcriptomic analysis in mouse brain tissue suggests they are a homogeneous cell population(241). Conversely, studies using immunohistochemistry have shown they are a heterogeneous cell population(242). RNA sequencing also suggests that pericytes do not express α-SMA(243) whereas other studies have shown they do(244). So far, the transcriptomic work has been mostly in mice; however, recent transcriptomic analyses in human tissue have identified putative novel human pericyte markers. A recent study by Song et al. used RNA-sequencing in mouse and human brain microvessel preparations to identify mouse-human species differences in vasculature-associated gene expression as well as build a putative gene expression profile of human brain pericytes. Some mouse-human differences included human brain pericytes express less VTN transcript (that encodes the extracellular matrix protein vitronectin) than mouse pericytes(245). Similarly, A2M, encoding α-2-macroglobulin is not expressed by mouse brain pericytes but single cell RNA-sequencing confirms its expression in human brain pericytes(245). In the same study, 186 putative pericyte genes were identified, including known pericyte markers PDGFRB, NOTCH3, ADGRA2, ABCC9 and S1PR3 and excluded endothelial cell genes (PECAM1, A2M and SLC2A1)(245). The authors that identified and mapped AD GWAS genes in the human vascular
system also identified two subtypes of human pericytes: transport- and matrix-pericytes, marked by distinct solute transporter, and extracellular matrix organisation gene transcripts respectively(125). Subsequent immunostaining revealed that transport- and matrix-pericyte markers are each present across both small- and large-diameter brain vessels. Their work suggested that human pericytes are transcriptionally more identifiable by their function than their location.

1.6.3 Pericyte function

Pericytes regulate BBB permeability, angiogenesis, neuroinflammation and neuroprotection, Aβ clearance and CBF (illustrated in Figure 10).

![Figure 10. Pericyte functions along the vasculature](image)

Simplified illustration depicting the various functions of pericytes along the vasculature i.e.: blood brain barrier (BBB) regulation, angiogenesis (formation of new blood vessels), immune function and phagocytic activity including the removal of Aβ, neurovascular coupling and blood flow regulation as well as multipotent stem cell activity. Image adapted from Zlokovic et al. 2016.

1.6.3.1 BBB regulation

Pericytes make direct contact and interact with endothelial cells via gap junctions and adherens junctions(246). This communication is an important regulator of BBB permeability. Early works demonstrated an inverse correlation between pericyte number and BBB leakage(247). More recent works, co-culturing pericytes with endothelial cells in vitro under normoxic conditions, have shown that the presence of pericytes promotes the formation of tight junctions on endothelial cells by stimulating the expression of the tight junction protein occludin(248, 249).
Pericytes also inhibit the production of molecules by endothelial cells that enhance vascular permeability (247, 250). Based on these pericyte-endothelial cell interactions, pericyte loss and/or migration away from endothelial cells would be expected to have negative consequences on vascular homeostasis. Mice with reduced pericyte number showed increased BBB leakage following reperfusion and increased transcytosis and immune cell extravasation as well as reduced number of functional tight junctions (247). *In vitro* work has also demonstrated minimal BBB damage and leakage when pericytes are near endothelial cells where they can signal via gap junctions (251). In disease models, pericyte loss and dysfunction have also been associated with accelerated Aβ pathology *in vivo* (252) through disrupted BBB permeability which has been found to worsen, particularly in the hippocampus and CA1 neurons, in association with MCI and early AD (224). From these findings, researchers have concluded that tight regulation of pericyte function, is important for maintaining BBB integrity and function and that pericyte loss and dysfunction are associated with AD. The exact molecular mechanisms that regulate the BBB remain largely unknown. Further investigation into understanding the pathways involved could provide potential opportunities for therapeutic intervention either by opening the BBB to deliver pharmacological agents or by reversing BBB breakdown in disorders like AD.

1.6.3.2 Angiogenesis

Angiogenesis is the formation of new capillaries from existing blood vessels (253). Bidirectional signalling and interaction between endothelial cells and pericytes are important in governing this process. Under hypoxic conditions induced by ischaemia, pericyte separation from the basement membrane and migration away from the vessel wall is seen under electron microscopy (254, 255). Thought to serve as a protective mechanism to avoid further pericyte damage following injury (255), migration may be to help guide other cells and promote vascular remodelling (256). For existing microvessels to begin remodelling, the vessel wall must disassemble. Pericyte detachment and migration are suggested to be important steps in allowing initial vessel disassembly to make room for new sprouting endothelial cells (257, 258). Toward the end of the angiogenic process, endothelial cells recruit pericytes via PDGF-B and PDGFRβ signalling (253, 259). Evidence in support of this comes from genetic knockout murine models in which heterozygous PDGF-B/PDGFRβ inactivation leads to reduced pericyte number. Suppression of this signalling pathway also leads to overexpression of vascular endothelial growth factor-A (VEGF-A), important in regulating vascular permeability and stabilisation. VEGF-A binds to VEGF-2 receptor (VEGFR-2) on endothelial cells and stimulates endothelial cell proliferation that can lead to hyperplasia if not tightly controlled. This signalling is opposed by
VEGFR-1/Flt1 in pericytes(259). Loss of pericytes and VEGFR-1 results in dysregulated endothelial sprouting and blood vessel formation. As well as PDGFRβ, human and murine pericytes are found to express functional Tie2 receptors, important in angiogenesis and blood vessel maturation(260). Deletion of Tie2 in pericytes resulted in delayed angiogenic processes in mice retina(260). Collectively, these data support an important role of pericytes in angiogenesis.

1.6.3.3 Immune function

Pioneering studies have demonstrated that pericytes are not mere bystanders in the immune response. Isolated pericytes display antigen-presenting cell-like characteristics, mimicking those of a macrophage, and exhibit phagocytic behaviour. IFN-γ has been shown to induce MHC class II mRNA(261) and protein expression(262) in pericytes. In a mouse model, PDGFRβ activation of pericytes induced expression of immunoregulatory genes including MHC class II molecules and Fc receptors(263). This work is yet to be replicated in human pericytes and calls for further study to establish the role of human pericytes as an antigen-presenting cell.

It is well known that endothelial cells can secrete a variety of immune molecules including cytokines and chemokines that help to mediate leukocyte recruitment during inflammation(264). Similarly, pericytes have been reported to release chemokine and cytokines such as CXCL8, IL-6, CCL2 and CCL3, in response to pro-inflammatory signals like TNF-α and IFN-γ(265).

Inflammatory molecules released by pericytes also have a key role in increasing vascular permeability during inflammation. TNF-α, IFN-γ and IL-1β promote the expression of nitric oxide synthase in the rat brain(266), which acts as a vasodilator and also increases vascular permeability, allowing leukocyte infiltration to the site of inflammation. Recent work has demonstrated that pericytes provide scaffolding for neutrophils to migrate along their processes (“abluminal crawling”) to gaps between adjacent pericytes which are enlarged at sites of inflammation to act as points of cell transmigration(267).

1.6.3.4 Stem cell-like and neuroprotective properties of pericytes

A recurring feature of pericytes in the literature is their capacity to act as stem cells and differentiate into other cell types. While the term pericyte is most used, some identify these cells as mesenchymal stem cells. Early works showed that pericytes act as a supplementary source of osteoprogenitor cells. During bone tissue damage, pericytes migrate, undergo morphological changes consistent with osteoblastoma formation and become incorporated into
the bone(268). Later works demonstrated pericyte differentiation into chondrocytes and adipocytes. One study revealed pericytes express mRNA for chondrocyte markers Sox9 and type II collagen and peroxisome proliferator-activated receptor-γ2 (an adipocyte-specific transcription factor). When cultured in chondrogenic or adipogenic medium, pericytes formed well-defined populations rich in type II collagen and adipogenic markers. Conversely, when endothelial cells were cultured under the same conditions, these cells failed to display chondrogenic or adipogenic differentiation(269). Similar findings show that pericytes extracted from ischaemic mouse brain tissue and human brain-derived pericytes grown under oxygen and glucose deprivation differentiate into cells of a vascular and neural lineage respectively(270). It is generally accepted that pericytes share features of mesenchymal stem cells but because both populations lack specific and definitive markers, it is difficult to determine whether they are one or two separate cell populations.

1.6.3.5  Aβ clearance

Pericytes have been suggested to play a role in the clearance of toxic products(235), displaying macrophage-like properties, exhibiting a phagocytic response to Aβ in AD(271). Previous studies have demonstrated that ApoE, specifically ApoE-ε4, disrupts Aβ clearance across the BBB in mouse models(272). One study has demonstrated that pericytes remove Aβ aggregates by an LRP1/APOE isoform-specific mechanism. This was shown by silencing APOE in pericytes in the presence of ApoE-ε3 or ApoE-ε4 which revealed ApoE-ε3 mediates LRP1-dependent removal of Aβ(273).

1.6.3.6  Blood flow and neurovascular coupling

VSMCs control CBF, through the constriction of arteries and arterioles in the brain. However, in vitro and in vivo studies of cortical brain slices and microcirculation, have demonstrated that the capillary network contributes most of the resistance within the cerebral circulation(274). Constriction of capillaries in human brain tissue is associated with pericyte contraction in areas of Aβ deposition(275), suggesting pericytes are the principal cell type involved in regulating blood flow at this level. Zlokovic et al. has assessed in vitro, ex vivo and in vivo studies on pericyte contractility and found that most of these studies reported that pericytes exhibit contractile properties(276).

Pericytes at pre-capillary arterioles, particularly those located at branching points, exhibit contractile properties(277, 278) and are believed to have a central a role in controlling CBF. Indeed, a subpopulation of CNS pericytes express α-SMA, a protein which is responsible for cell
contractility as shown in studies examining pericytes found in retinal capillaries from mice(279). This supports previous studies that have shown abundant α-SMA expression in cells surrounding ‘sphincters’; points at precapillary branch points that are involved in controlling CBF. This study demonstrated that the precapillary sphincters are capable of generating large changes in CBF resistance, thereby controlling capillary blood flow(280). Recent optogenetic activation of these pericytes also shows these cells can regulate capillary blood flow and diameter and that this activity could be inhibited using the vasodilator, Fasudil(281).

A recent study investigating the RNA profile of pericytes revealed expression of genes which encode other contractile proteins including desmin, calponin-2, and myosin light chain-9(231); however, the extent of their involvement in pericyte contraction is still to be determined. Pericyte loss has been shown to lead to neurovascular uncoupling and reduced oxygen supply to the brain(282), as well as diminished CBF in grey matter suggesting a central role of pericytes in regulating cerebral microcirculation(235, 283).

1.6.4 Pericytes in Alzheimer’s disease

Abnormalities in the ultrastructure of cortical pericytes in AD have been observed by electron microscopy. These include mitochondrial alterations and lipid granulation(284). Sengillo and colleagues showed a decrease in pericyte number in AD brains compared to non-demented controls. Pericyte number and coverage in the cortex and hippocampus were reduced by two thirds and one third respectively and the reduction in coverage correlated with the extent of BBB breakdown(285). The level of PDGFRβ (a pericyte marker), measured by ELISA, was reduced in AD and was inversely correlated to fibrinogen level (a proxy marker of BBB breakdown) in the precuneus in a human pathological study(286). Pericyte loss as evidenced by reduced PDGFRβ, was more marked in ApoE-ε4 individuals(286). PDGFRβ can be released in a soluble form, via ADAM-10 and ADAM-17 mediated cleavage, from injured pericytes exposed to hypoxia and/or Aβ peptides(287, 288). Levels of soluble PDGFRβ (sPDGFRβ), are elevated in CSF in AD(289). sPDGFRβ level positively correlated with CSF albumin, t-tau and p-tau, which are CSF markers of BBB integrity and AD pathology, respectively(289). This suggests an association between pericyte injury and BBB breakdown. sPDGFRβ and hippocampal-specific BBB leakiness have been demonstrated in normal ageing and MCI(224) and occurs at the very earliest stages of AD(290). Increased CSF sPDGFRβ and regional BBB leakiness in the early stages of AD predict cognitive decline independently of CSF-Aβ or tau(290). These findings are in keeping with the data from PDGFRβ-deficient mice(289), further supporting that pericyte loss and BBB leakiness contribute to cognitive decline and AD.
The timing and cause of pericyte dysfunction are unclear but accelerated pericyte degeneration in AD underlying BBB breakdown is ApoE-ε4 specific(291). Neurovascular uncoupling was observed in APP mice before amyloid pathology and cognitive decline(199), indicating that functional changes within the neurovascular coupling are an early-disease-stage phenomenon in AD. Studies showing BBB dysfunction in the hippocampus in normal ageing and MCI(224, 290) indicates that BBB leakiness is an abnormality detected in very early stages in AD.

The mechanisms of pericyte loss in AD are still undetermined. In normal ageing, some animal studies have suggested that the number of pericytes in the brain increase(292) whilst some suggest no change(293). Initial data suggested that vascular factors like dyslipidaemia and hypertension may lead to pericyte death(294, 295); however, additional investigation is still needed to establish the causative link between vascular dysfunction and pericyte loss in early AD. In mice overexpressing ApoE-ε4, proinflammatory cyclophilin A-nuclear factor κB-MMP9 pathway was upregulated within pericytes. MMP9 released from pericytes becomes activated, which leads to pericyte-endothelial tight junction degradation resulting in BBB breakdown(296). Under pathological conditions, pericytes are susceptible to oxidative stress(297) resulting from an overproduction of reactive oxygen species released by mitochondria(297). Ding et al. has demonstrated pericyte death because of reactive oxygen species production by activated microglia(298).

In late stage disease, Aβ accumulates on and around small blood vessels and on pericytes(299). Pericytes express LRP-1 which allows Aβ to bind and internalise for lysosomal degradation(300). Chronic exposure of elevated Aβ in the brain impairs the pericytes capability to internalise and clear Aβ, leading to subsequent injury and loss of pericytes(252). Other works suggest a role of CD36, an innate immunity receptor involved in Aβ trafficking, in AD-related pericyte death. One study showed CD36 deficient mice demonstrated a selective reduction in Aβ1-40 levels which was associated with preservation of LRP-1(301).

Pericyte degeneration can influence AD-like neurodegeneration and contribute to pathogenesis. In PDGFRβ-deficient mice, pericyte loss was associated with elevated brain Aβ1-40 and Aβ1-42 as well as accelerated amyloid angiopathy, cerebral amyloidosis (from a reduction in clearance), increased tauopathy, neuronal loss and accelerated cognitive decline(252). In animal models, pericyte loss can lead to neurodegenerative changes independent of amyloid. Pericytes loss increases vascular permeability through disrupted BBB integrity. This in turn leads to an abnormal accumulation of fibrin, thrombin and reactive oxygen species that are toxic to the
human brain(302). Simultaneously, pericyte loss also leads to brain capillary regression, giving rise to perfusion deficits, vascular stress, and hypoxia(283, 303).

Many studies have sought to define the mechanisms underlying pericyte-mediated cerebral hypoperfusion in AD. Some studies have shown that following a stroke, pericytes constrict capillaries and die in rigor(304) causing long-lasting restriction of blood flow, also known as ‘no-reflow’(305). These changes in pericytes after stroke are related to an abnormal influx of intracellular calcium and oxidative stress(304) which may also apply to AD. A recent study published by Notley et al showed vasoconstriction of the capillary bed in proximity to contracted pericytes in the cortex in areas with high Aβ peptide load in human resected brain tissue. Mechanistically, oligomeric Aβ₁₋₄₂ peptide was shown to induce pericyte contraction via oxidative stress pathways leading to overproduction of EDN1 in rodent studies(275).

As mentioned above, vascular dysfunction and cerebral hypoperfusion occur years before Aβ accumulation(33, 306). Therefore, accumulated insoluble Aβ cannot be solely responsible for bringing about this perfusion deficit. Works by Palmer and colleagues have demonstrated an increase in endothelin-1 (EDN1), a potent vasoconstrictor, in the brain during AD(307). Using the myelin-associated glycoprotein (susceptible to hypoxia): proteolipid protein-1 (resistant to hypoxia; MAG: PLP1) ratio as an indicator of oxygenation of brain tissue during life, Thomas and colleagues revealed a significant reduction in MAG: PLP1 ratio in the frontal cortex of AD patients with Braak tangle stage III-IV disease. The results suggested a reduction in oxygenation in the cerebral cortex in early AD (308). This same group also reported a significant correlation between reduced MAG:PLP1 and increased EDN1 concentration(206). Their data revealed that the cerebral perfusion deficit was not simply a consequence of reduced metabolic demand due to neuronal death at end-stage disease. Numerous functional MRI studies have collectively shown that oxygen extraction is increased (rather than decreased) in AD(309, 310). If blood flow reduction were due to reduced metabolic demand, a reduction in oxygen extraction would be expected. Together, these data suggest that cerebral hypoperfusion is an early disease stage phenomenon in AD contributed to by elevated EDN1.

1.1 Endothelin-1

EDN1 is a potent vasoconstrictor peptide. It is the most common human isoform of the endothelin peptides. These also include EDN2 and EDN3, which are less abundant and not as well studied(311, 312). The gene encoding EDN1 is found on chromosome 6(313). Synthesis of
EDN1 involves a series of enzymatic cleavages starting with a 212 amino acid long prepro-EDN1. Proteases acting in the endoplasmic reticulum cleave prepro-EDN1 to a 38 amino acid long peptide called big EDN1. The final cleavage step is mediated by endothelin converting enzyme (ECE) which convert big EDN1 into active EDN1(314). EDN1 is produced in a variety of cells: however, endothelial cells express high levels of mRNA for EDN1’s precursor prepro-EDN1 as well as ECE and are therefore thought to be the main site of EDN1 production(315, 316).

EDN1 has a wide range of biological actions. It was originally identified in 1988 as an endothelium-derived factor that brought about vasoconstriction and a subsequent increase in arterial blood pressure(317). Studies since have revealed a role of EDN1 in cancer biology, immune function, and embryogenesis(318). The diverse actions of EDN1 are mediated mainly via two transmembrane G-protein coupled receptors (GPCRs): endothelin receptor type-A (EDNRA) and endothelin receptor type-B (EDNRB). EDNRAs are predominantly found on mural cells including VSCMs but are also found on cardiomyocytes, fibroblasts, and neurons. EDNRBs are highly expressed on endothelial cells as well as SMCs and neurons(319). It is generally accepted that binding of EDN1 to EDNRA brings about vasoconstrictor effects, while binding of EDN1 to EDNRB brings about vasodilatory ones(319). The various other signalling events through activation of these receptors are nicely summarised elsewhere(320). Palmer and colleagues showed EDN1 synthesis was increased in AD. Their work showed that the two enzymes involved in its production were upregulated in the presence of Aβ peptides. ECE-1 was upregulated by Aβ1-40(321); ECE-2, produced by neurons, was significantly upregulated by Aβ1-42(322). A recent study demonstrated that elevated EDN1 leads to learning and memory impairment. Mice trained on the Morris water maze showed significantly increased escape time and considerably poorer spatial awareness and associative memory compared to saline-treated mice(323). These findings suggested possible involvement of vasoconstrictor effects and downstream pathways that drive behavioural abnormalities.

These data, alongside existing literature, indicate that pericytes are important in regulating CBF and neurovascular coupling, and that dysfunction and degeneration of pericytes, mediated in part by elevated EDN1 levels, contribute to neurovascular uncoupling leading to cerebral hypoperfusion in the early stages of AD.

1.2 Hypothesis and aims

There is evidence that vascular dysfunction, specifically neurovascular uncoupling, and BBB damage, are major contributors to cognitive decline and disease progression in the early stages
of AD. Reductions in CBF in AD are associated with pericyte dysfunction leading to neurovascular uncoupling and capillary constriction possibly associated with elevated expression of EDN1. In this dissertation, I will explore the hypothesis that abnormal EDN1-evoked pericyte contraction is a contributor to cerebral hypoperfusion in AD.

To address this hypothesis, the aims of this research are to:

1. Characterise the EDN1-mediated contractile response of human brain-derived vascular pericytes in vitro
2. Investigate the effects of Aβ on the EDN1-mediated contractile response in vitro
3. Map the distribution and disease-related changes in the gene and protein expression of EDNRA and EDNRB in cell models and human post-mortem brain tissue.
4. Investigate the relationships between endothelin receptor expression and markers of vascular dysfunction, including markers of cerebral hypoperfusion and BBB breakdown, and disease pathology in AD in human post-mortem brain tissue.
# Chapter 2  Materials and Methods

## 2.1  Materials

### 2.1.1  Consumables

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<thead>
<tr>
<th>Item</th>
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<td>Albumin standard</td>
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### 2.1.2 Equipment

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<td>Bench top microscope</td>
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## Solution constituents

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<td>1% SDS lysis buffer (diluted 1 in 10 from 10% stock)</td>
<td>10% SDS (w/v): 10 g in 100 ml $dH_2O$, 5M NaCl: 29.22 g NaCl in 100 ml $dH_2O$, 1M Tris-HCL (Trizma-HCl): 12.1 g in 100 ml $dH_2O$, pH 7.6</td>
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<td>Citrate Buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0)</td>
<td>Citric acid (1.92 g), dH2O (1 L), pH 6.0 then add 0.5 ml Tween 20</td>
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## Primary antibodies

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<td><strong>Anti-Endothelin B Receptor Antibody</strong></td>
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<td><strong>Endothelin A receptor antibody</strong></td>
<td><strong>Rabbit polyclonal</strong></td>
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<td><strong>Human PDGFR Beta Antibody</strong></td>
<td><strong>Goat polyclonal</strong></td>
<td><strong>Mouse myeloma cell line NS0-derived recombinant human PDGF R beta Leu33-Phe530 (Glu241Asp) Accession # P09619</strong></td>
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### Secondary antibodies

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<td>Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 555</td>
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<td>Invitrogen (A21206)</td>
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<td>Streptavidin, Alexa Fluor 555 conjugate</td>
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### Assay Kits

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<tr>
<td>BrdU Cell Proliferation ELISA Kit (colometric)</td>
<td>Abcam (ab126556)</td>
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<tr>
<td>High-Capacity cDNA Reverse Transcription Kit</td>
<td>ThermoFisher (4368814)</td>
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<tr>
<td>Human ETRA ELISA Kit</td>
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<tr>
<td>TaqMan® Fast Advanced Cells-to-Ct™ Kit</td>
<td>Invitrogen (A35374)</td>
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2.1.7 PCR primers

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<td>Collagen IV</td>
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<td>Ubiquitin-conjugating enzyme E2 D2</td>
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2.1.8 Cell lines

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<tr>
<td>Human brain vascular pericytes (foetal)</td>
<td>ScienCell (1200)</td>
</tr>
<tr>
<td>Primary human brain pericytes (adult)</td>
<td>Cell-systems (ACBRI 498)</td>
</tr>
</tbody>
</table>

Human brain-derived vascular pericytes of foetal origin (fHBVP; ScienCell, USA) and human brain-derived vascular pericytes of adult origin (aHBVP; Cell Systems, USA) were grown in pericyte media (ScienCell, USA) and incubated at 37°C in 5% CO2. All experiments were performed at passages of < 8. Cells were cultured in poly-L-lysine-coated flasks and maintained in media until 80% confluent and then sub-cultured according to standard cell culture techniques. All work was performed in a class II microbiological safety cabinet (Holten, UK) under sterile conditions.

2.1.9 Statistical software

GraphPad Prism 8.1.2 and IBM SPSS Statistics 24 software packages were used for statistical analyses. All data sets were analysed by Shapiro-Wilk normality test to determine whether the data was normally distributed. A p-value < 0.05 (unless otherwise stated in individual chapters) was considered to vary significantly from normality and the data were considered not-normally distributed. One-way ANOVA, Pearson’s and two-tailed unpaired t-tests were used for normally distributed data or data that was normally distributed after log-transformation. Kruskal-Wallis,
Spearman’s and Mann-Whitney U tests were used for datasets that were not normally distributed after log transformation. Additional two-way ANOVAs have been used to assess differences between foetal-adult and $\Lambda^\beta_{1.40}$-$\Lambda^\beta_{1.42}$ contractile responses and paired analyses (RM-one-way ANOVA and Friedman test) have also been performed to account for between experimental variation.

### 2.2 Methods

#### 2.2.1 Cell culture

Cell culture is the process by which live cells are taken from a living organism and grown and manipulated in a controlled laboratory environment. Cells serve as a useful model for studying complex physiological processes and so are commonly used in research. Details of the specific cell lines used in these cell culture experiments are outlined in section 2.1.8. Described below is the general approach used to culture cells in a sterile environment including starting and maintaining cultures as well as measures put in place to minimise contamination. The experimental manipulations of cells in various experiments are outlined in later chapters.

#### 2.2.1.1 Cell culture laboratory

Culturing of cells was performed in a class II microbiological safety cabinet (Thermo Scientific, UK) that uses a combination of downward and inward airflow, taking air from outside and passing it over high-efficiency particulate air (HEPA) filters, to ensure a steady flow of sterile air over the cells to minimise risk of contamination to the user. The cabinet also houses a built-in UV lamp that was turned on after every use for 2 hours to ensure a sterile working environment. Before starting work, the inside of the cabinet as well as gloves and all equipment being placed inside was sprayed with 70% ethanol to reduce risk of contamination to both user and cell cultures. The cabinet was kept clear of materials and equipment to ensure uniform airflow. Used pipette tips and liquid waste was treated with disinfectant (e.g., Virkon®) before being discarded.

Contamination of cell cultures can affect cell growth and behaviour and thus could potentially affect experimental results. Cell cultures were checked regularly by eye under an upright phase contrast microscope (Olympus) for common indicators of contamination such as a change in colour of the media (may turn cloudy or have a yellow/orange tinge), or a change in cell morphology that is not consistent with normal cell growth. Monthly mycoplasma testing using
a Promokine PCR mycoplasma I/C kit (PromoCell, Germany) was also performed to ensure cell cultures were clear of mycoplasma (undertaken by the technical support team). Mycoplasmas are small bacteria with no cell wall and so are invisible to the naked eye. They can adhere to cell surfaces, infect and can seriously affect cell growth, proliferation and behaviour (324).

Any infected cell cultures were disposed of immediately, affected culture vessels were treated with disinfectant before being discarded. To prevent spread or further contamination, all potentially contaminated areas in the laboratory were also cleaned with disinfectant.

### 2.2.1.2 Cell growth environment

Different cell lines require different media which usually contain essential salts, amino acids, and growth factors as well as antimicrobial compounds. Media is designed to provide a defined balanced nutritional environment for optimal growth and proliferation of selected cell types *in vitro*. Pericyte medium *(ScienCell, USA)* containing foetal bovine serum, pericyte growth supplement and penicillin/streptomycin solution was used for optimal growth of both fHBVP and aHBVP used herein.

Different vessels can be used to culture cells. Cell culture flasks *(Greiner Bio-One, Austria)*: T25; surface area of 25 cm² or T75; surface area of 75 cm² were used to grow and maintain cell cultures. 4- to 24-well plates were used to hold 100 x 13 mm diameter coverslips *(VWR, USA)* onto which cells were seeded and grown for immunocytochemistry (see section 2.2.2). All culture vessels and coverslips were precoated with poly-L-lysine before cells were seeded (see section 2.2.1.3). This coating facilitates and promotes the attachment of cells to the solid surface of flasks, wells, and coverslips by improving the electrostatic interaction between the positively charged surface ions of the culture vessel and the negatively charged ions of the cell membrane.

### 2.2.1.3 Flask and coverslip sterilisation and preparation

Flasks were coated by adding 5 µl poly-L-lysine to 5 ml PBS for a T25 flask and 15 µl to 10 ml PBS for a T75 flask. Flasks were then left in a 37°C, 5% CO₂ incubator for a minimum of 2 hours or overnight. Poly-L-lysine was removed, and the bottom of the flask was washed x2 with PBS. Flasks were then sealed and stored at 4°C for long term storage.

To sterilise and coat coverslips, 100 coverslips were placed in a 50 ml centrifuge tube filled with 30-40 ml ethanol and placed on a roller for a minimum of 2 hours. Ethanol was aspirated off and coverslips were washed in the fume hood x3 with dH₂O. The tube was inverted each time to break up the coverslips to stop them from sticking to each other and ensure thorough washing.
Coverslips were placed in between two sheets of round filter paper in a glass petri dish and sealed with autoclave tape ready to be autoclaved. The dish with coverslips was then placed in the 37°C oven overnight to dry. The dish containing the coverslips was transferred to the fume hood and individual coverslips were transferred using forceps into individual wells of a 4- or 24-well plate. Coverslips were then coated with poly-L-lysine 5 mg/ml and left to incubate at 37°C in a 5% CO₂ incubator for 2-24 hours. Poly-L-lysine was removed, and coverslips were washed x2 in PBS before being sealed and stored at 4°C for long term storage ready for use.

2.2.1.4 Starting new cell cultures

Media was prewarmed to 37°C in a water bath to prevent disruption of cells by sudden changes in temperature. 10-12 ml of warmed media was added to a new poly-L-lysine-coated T75 flask. In the hood, 5-8 ml of warmed media was added to a 15 ml falcon tube. A new vial of cells was taken from storage, sprayed with 70% ethanol, and thawed in the water bath. Once completely thawed, the vial was sprayed again with 70% ethanol before being placed in the hood. The contents of the vial were transferred to the falcon tube and the resulting cell suspension was centrifuged at 500 x g for 4 minutes to pellet the cells. Supernatant was discarded and the cell pellet was resuspended in 2 ml of media and transferred to the flask. The contents of flask were viewed under the microscope to confirm the presence of cells. The flask was then placed in a 37°C 5% CO₂ incubator to grow until next required.

2.2.1.5 Sub-culturing cells

Sub-culturing cells is a process by which a small number of cells from a near-confluent flask are transferred to a new flask to continue growing, avoiding senescence because of overcrowding. Cells were sub-cultured when approximately 80% confluency was reached. Pericyte medium, PBS and accutase solution (Sigma-Aldrich, USA) were prewarmed in a 37°C water bath before use. Old medium was removed, and the flask was washed with 6 ml PBS, ensuring the whole bottom surface of the flask was covered. The PBS was then removed and 2 ml of accutase was added and left for 5-10 minutes. The flask was gently agitated to encourage cells to detach from the bottom of the flask and checked regularly under the microscope. When cells were detached and in suspension, an equal volume of media was added, taken up and dispensed back over the bottom of the flask to ensure all the cells were detached. Resulting solution was transferred to a 15 ml centrifuge tube. Cells were centrifuged for 4 minutes at 500 x g. The supernatant was removed, and cells were resuspended in 2 ml fresh medium. A specific volume of the resulting cell suspension was taken and transferred into a new flask and 10 ml of new medium was added.
and left to incubate at 37°C in a 5% CO₂ incubator until next required. HBVP were sub-cultured no more than 8 times.

2.2.1.6 Cell counts

Cell counts were obtained using 0.4% trypan blue solution (Sigma-Aldrich, USA) which stains only non-viable (dead) cells. 100 µl cell suspension was taken and diluted 1:1 with trypan blue.

Cells (per mL) = Mean cell count (per square) × Dilution factor (2) × Chamber conversion factor (10⁴)

The coverslip was placed onto the haemocytometer and both chambers were filled with trypan blue treated cell suspension. Counts were taken from the four, 4 by 4 corner squares (unstained; viable cells) and an average was taken. This was done for both chambers and the averages of both chambers were used. The final cell count was calculated using the following equation:

Cell count was repeated if there was a 10% difference between the averages of the two chambers or if there were large clusters of cells within each chamber.

2.2.1.7 Cryoprotection of cells

Cryopreservation of cells allows long-term storage of cell stocks. Cells were sub-cultured as detailed in section 2.4.6. Supernatant was removed and cells were instead resuspended in freezing medium: 50% foetal bovine serum, 40% pericyte medium and 10% dimethyl sulphoxide (DMSO; Sigma, UK). 1 ml aliquots were transferred to cryovials which were placed into an isopropanol-containing freezer container (“Mr Frosty”, Nalgene Labware, UK) and stored overnight at -80°C before being transferred to -150°C for long term storage.

2.2.2 Immunocytochemistry

Immunofluorescence was used to confirm expression and determine distribution of target proteins in cell cultures. Cells were grown on poly-L-lysine coated coverslips until desired confluency was achieved (~60%). Media was then aspirated off and cells were gently washed x2 with PBS. Cells were then fixed in 4% paraformaldehyde (Sigma-Aldrich, USA) for 10 minutes, incubated at 37°C in a 5% CO₂ incubator. Cells were then washed a final x2 in PBS and left in its final wash at 4°C until immunolabelled. Cells were washed with cold methanol for 10 minutes before being washed with PBS x3 for 1 minute each and then blocked with 5% donkey serum (Sigma-Aldrich, USA) for 30 minutes at room temperature. Cells were washed again in PBS x3 for 1 minute each and incubated with a primary antibody diluted to an assay-dependent concentration overnight at 4°C. Cells were washed again in PBS x3 for 1 minute each followed
by addition of a secondary antibody (1:500) and left to incubate for 1 hour at room temperature in the dark. Cells were washed a final time in PBS x3 for 1 minute each and coverslips were removed and placed onto glass microscope slides with Vectashield mounting medium with DAPI (Vector Labs, USA). Cells were stored at 4°C in the dark. See specific assay methods for details of primary and secondary antibodies.

2.2.3 Electrical impedance assay (xCELLigence)

An electrical impedance assay was used to detect changes in total contact surface area between the pericytes and the culture dish using an xCELLigence® RTCA S16 or SP96 (Agilent Technologies Inc, USA). The machine was maintained at 37°C in a 5% CO₂ incubator. fHBVP and aHBVP were seeded at 5000 cells/well onto pre-coated poly-L-lysine impedance plates (E-plate VIEW® 96 or E Plate insert 16®, Agilent Technologies Inc, USA) and allowed to adhere for 1 hour at room temperature within the hood and then overnight in the incubator at 37°C, 5% CO₂. Cells were then starved in serum-free media 2 hours prior to compound addition. Compounds were added in situ and measurements were continuously recorded throughout. Parameters for the xCELLigence software were set to record in 5 second intervals for 2 hours to measure contraction, and then in 1-hour intervals for 48 hours to measure proliferation. Addition of serum-free media was used as a vehicle control. Impedance data is presented as a “cell index” (an arbitrary value derived from the impedance software) representing contact surface area between cells and the culture dish (i.e., resistance to the electrical current; Figure 11). Contraction and relaxation data are presented as calculated change in slope between two timepoints on the cell index curve (built in function of the data analysis software; represented as 1/ hour). These data are normalised to baseline (where cell index is 0) wells that contain cells and serum free media only. Specified segments of the cell index profile that reflect contractile and relaxation phases have been graphed separately and highlighted. Data analyses were performed using RTCA Data Analysis Software 1.0 (Agilent Technologies Inc, USA). Measurements were recorded in duplicate wells for each condition; ‘n’ refers to the number of independent experimental repeats.
Cell viability assay

A LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen, UK) was used to assess cell viability. The LIVE/DEAD® Viability/Cytotoxicity Kit discriminates live from dead cells by simultaneously staining with green-fluorescent calcein-AM to indicate intracellular esterase activity in live cells with an intact plasma membrane and red-fluorescent ethidium homodimer-1 to indicate loss of plasma membrane integrity. Cells were seeded at 5,000 cells/well onto precoated poly-L-lysine
coverslips and allowed to adhere for 10 minutes at room temperature and then for 24 hours at 37°C, 5% CO₂. When cells reached 80% confluency, the drug was added in situ prior to or concurrent with LIVE/DEAD® reagent staining (see specific assay methods for treatment administration and incubation times). Media was removed and 100-150 µl LIVE/DEAD® assay reagent was added to each well and covered to prevent drying. Cells were left to incubate for 30-45 minutes at room temperature. The fluorescence was measured using a FLUOstar OPTIMA microplate reader using the appropriate excitation and emission filters according to the manufacturers protocol.

2.2.5 BrdU assay

A BrdU Cell Proliferation ELISA kit (Colourmetric) (Abcam, UK) was used to investigate cell proliferation and viability. Cells were seeded onto a poly-L-lysine precoated 96 well plate at a cell density of 5000 cells per well and left to adhere for 24 hours. Test reagent (EDN1) was then added in 100 µl media. 1X BrdU was then added to the appropriate wells and incubated for 18 hours. The media was removed and 200 µl Fixing Solution was added to cells and incubated at room temperature for 30 minutes. The Fixing Solution was removed and the plate was inverted and patted dry. Wells were then washed x3 with 1X wash buffer to prepare the wells for BrdU detection (following manufacturer’s instructions). The final wash solution was removed and the plate was inverted and patted dry. 100 µl anti-BrdU monoclonal detector antibody was added and incubated for 1 hour at room temperature. The plate was washed again x3 with 1X wash buffer. 100 µl 1X peroxidase Goat Anti-Mouse IgG Conjugate was added and incubated for 30 minutes at room temperature. The plate was washed a final x3 and then flooded with dH₂O before being inverted and patted dry. 100 µl TMB Peroxidase substrate was added and incubated for 30 minutes at room temperature in the dark. 100 µl Stop Solution was then added and the plate was read at 450 nm. Control wells were run in parallel, one with only media added and one with cells present but in the absence of the BrdU Reagent. All treatment concentrations were repeated in triplicate.

2.2.6 Real-time polymerase chain reaction (PCR) of cell culture samples

The relative expression levels of gene transcripts (messenger RNA; mRNA) were measured using Reverse transcription (RT)-PCR. The TaqMan® Gene Expression Cells-to-CT™ Kit (ThermoScientific, UK) was used to isolate RNA direct from cell lysates and then for synthesis of cDNA.
2.2.6.1 Cell lysis using Cells-to-CT™ kit

Cells were cultured (10-10⁵) onto a precoated poly-L-lysine 96-well plate and allowed to adhere for 1 hour at room temperature and then for 24 hours at 37°C, 5% CO₂. Cells were washed with cold PBS and gently mixed x5 with Lysis Solution and incubated at room temperature for 5 minutes. 5 µl of Stop Solution was then added to each lysate reaction, mixed by pipetting up and down x5 and incubated at room temperature for 2 minutes. Samples were then stored at -80°C until required.

2.2.6.2 RNA quantification

Total RNA was quantified using a Qubit RNA High Sensitivity assay (ThermoFisher, UK). Quant-iT™ Working Solution was prepared by diluting the Quant-iT™ reagent in Quant-iT™ buffer (1:200) and 190 µl of this working solution was distributed to each standard along with 10 µl standard solution. Working Solution (195 µl) was added to each sample tube followed by 5 µl of sample. Standards and samples were vortexed for 2-3 seconds and incubated at room temperature for 2 minutes in the dark. Samples were read in duplicate using a Quibit™ fluorometer and the average reading was taken.

2.2.6.3 Reverse transcription (RT) using Cells-to-CT™ kit

The RT Master Mix was prepared in nuclease-free microcentrifuge tubes on ice for the number of reactions required plus 10% overage according to the manufacturer’s protocol. The solutions were mixed gently but thoroughly, briefly centrifuged and then placed on ice. The Master Mix was distributed to nuclease-free PCR tubes or wells of a sterile multi-well plate. Cell lysates were added to each aliquot of the RT Master Mix, gently mixed and then centrifuged briefly. An RT-minus control was also run in parallel in which the enzyme mix was replaced with nuclease-free H₂O. The thermal cycler (GeneAmp® PCR System 9700; Applied Biosystems, USA) was set up to run using the following settings: Reverse Transcription (hold) 37°C for 30 minutes, Reverse Transcription inactivation (hold) 95°C for 5 minutes and then held at 4°C. Completed RT reactions were then stored at -80°C until required.

2.2.6.4 Measurement of gene expression by real-time PCR

The PCR Cocktail was prepared in a nuclease-free microcentrifuge tube on ice (plus 10% overage) according to the manufacturer’s protocol; 5 µl TaqMan® Fast Advanced Master Mix, 0.5 µl TaqMan® Primer Assays and 2.5 µl nuclease-free dH₂O. The PCR Cocktail was distributed into individual PCR tubes or wells of a real-time PCR plate. 2 ng/ml cDNA was added to the PCR
Cocktail in each well to make a final reaction volume of 10 µl. Samples were gently mixed with the tube closed, and briefly centrifuged. All samples were run in triplicate including a non-template control in which cDNA was replaced with nuclease-free dH₂O. The plate was sealed and the PCR reactions were run using the real-time PCR system, ViiA7™ (Applied Biosystems, USA) as per the following: UDG activation (1 x cycle at 50°C for 2 minutes); enzyme activation (1 x cycle at 95°C for 20 seconds); PCR (40 x cycles at 95°C for 1 second and 60°C for 20 seconds).

2.2.6.5 Interpretation of results

Results of real-time PCR are given as C_q values, where C_q reflects the number of PCR cycles at which amplified gene product is first detected (the C_q value is inversely proportional to the amount of gene product). Data is presented as comparative C_q (2^{-\Delta\Delta C_q}) whereby the expression of a target gene is presented as a fold change relative to a control group. It is calculated using the following method: the mean C_q (from triplicate samples) was calculated for target genes in each sample as well as two reference (housekeeping) genes: RPL13 and UBE2D2 to which the data was normalised. Mean C_q values for the reference genes were subtracted from the mean C_q value of the target gene which calculates \Delta C_q. The \Delta C_q value of the control was then subtracted from the \Delta C_q value of the sample to give the \Delta\Delta C_q value. The fold change was then expressed as comparative fold changes in gene expression relative the control sample (2^{-\Delta\Delta C_q}).

2.2.7 Processing and handling of human brain tissue

This study was conducted under Human Tissue Bank ethics approval. Brain tissue from AD and age-matched controls were sourced from the South-West Dementia Brain Bank (SWDBB; Human Tissue Authority licence 12271), University of Bristol. All brains had undergone extensive neuropathological assessment in agreement with the National Institute on Aging-Alzheimer’s Association guidelines(325). AD pathology was sufficient for dementia diagnosis. Criteria for selection of control brains were few or no neuritic plaques, Braak stage of III or less and no additional neuropathological abnormalities. Before processing and formation of paraffin wax (Surgipath, UK) blocks, the right cortex was fixed in 10% formalin for 3 weeks minimum. Table 1 shows the summary data of the cohort of post-mortem brain tissue used in this study. See Appendix Table 1 - Appendix Table 2 for full summary of demographic and neuropathological data of control and AD cohorts.
### Table 1. PM brain tissue cohort demographic summary

Summary of the demographic statistics of the Control and AD cohorts used in this study. Where applicable, the mean ±SD are given. N = number.

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<th>AD</th>
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</thead>
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<td>25</td>
</tr>
<tr>
<td>Age (±SD)</td>
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<td>77 (±7)</td>
</tr>
<tr>
<td>Post-mortem delay (hours) (±SD)</td>
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</tr>
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</tr>
<tr>
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<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

#### 2.2.8 Immunofluorescent labelling of FFPE brain tissue

Immunofluorescent labelling was carried out on sections of FFPE parietal cortex (7 μm) to stain for receptor expression in control and AD cases. The sections were mounted onto 3-aminopropyltriethoxysaline-coated slides and left to dry overnight at 40°C. Prior to staining, the sections were incubated overnight at 65°C to remove the wax before being washed in clearene x2 for 5 minutes and then washed in ethanol x2 for 5 minutes. The sections were then washed in running water for 10 minutes before antigen retrieval pre-treatment (see specific details in respective results chapters) followed by another wash in running water for 10 minutes. The sections were then washed in PBS for 5 minutes before applying normal donkey blocking serum diluted in PBS (5%) for 30 minutes which were then drained, washed in PBS x4 for 3 minutes each and primary antibody was applied and left to incubate in the fridge (4°C) overnight. The sections were washed in PBS x3 for 3 minutes each before a secondary antibody was applied and incubated for 1 hour in the dark at RT. The sections were then washed in PBS x4 for 5 minutes before being washed in ethanol (70%) for 5 minutes. Autofluorescence Eliminator Reagent (*Merck, Germany*) was applied to the sections for 5 minutes before being washed again in ethanol (70%) x3 for 1 minute each. The sections were washed a final time in PBS for 5 minutes before being mounted onto coverslips with Vectashield mounting medium with DAPI (*Vector Labs, USA*). The sections were stored at 4°C in the dark before being visualised using...
fluorescence or confocal microscopy (*Nikon eclipse 80i*). See specific assay methods for details of primary and secondary antibodies as well as antigen retrieval process.

2.2.9 Quantifying immunofluorescence in human post-mortem FFPE brain tissue

With the help of Dr Stephen Cross from the Bioimaging facility at the University of Bristol, a software plug-in for ImageJ was created and developed to isolate positively stained pericytes on microvessels in human post-mortem FFPE brain tissue sections. Areas of interest were segmented and the average fluorescence intensity was measured and used to calculate the mean level of fluorescence for each image. The average fluorescence across 5 images was calculated and used to reflect receptor expression for each case.

2.2.10 RT-PCR of human tissue samples

2.2.10.1 RNA extraction from frozen brain tissue

Frozen brain tissue from the parietal cortex was dissected (70 mg) and stored at -80°C until used. A RNeasy Lipid Tissue Mini Kit (*Qiagen, UK*) was used to extract RNA from frozen brain tissue. 5 zirconia/silica beads and 1 ml QIAzol lysis buffer were added to each sample and were homogenised for x2 30 seconds in a Precellys 24 Homogeniser. In a class II safety cabinet, 1 ml of homogenate was transferred to microcentrifuge tubes and incubated at room temperature for 5 minutes. 200 µl chloroform was added to the samples and shaken vigorously to mix for 15 seconds and then incubated at room temperature for 3 minutes. Samples were then placed in a refrigerated centrifuge and centrifuged at 4°C for 15 minutes at 12,000 g. 520 µl of the resultant aqueous phase was transferred to a new microcentrifuge tube and an equal amount of 70% ethanol was added and vortexed briefly to mix. Half of the mixture was transferred to a spin column in a 2 ml collection tube and centrifuged at room temperature for 15 seconds at 8,000 g. The flow through was discarded and the second half of the mixture was transferred to the same spin column, centrifuged again at room temperature for 15 seconds at 8,000 g and the flow-through was discarded. 350 µl RW1 buffer was added to the spin column, centrifuged at room temperature for 15 seconds at 8,000 g. The flow-through was discarded. DNase I mix was prepared (10 µl + 10% DNase I stock added to 70 µl + 10% buffer RDD) and added to each sample onto the membrane and incubated for 15 minutes at room temperature. 350 µl RW1 buffer was then added to each sample and centrifuged at room temperature for 15 seconds at 8,000 g. The flow through was discarded. 500 µl RPE buffer was added to each sample, centrifuged at room temperature for 15 seconds at 8,000 g, discarding the flow-through. A second 500 µl was added to each sample centrifuged at room temperature for 2 minutes at
8,000 g. The spin column was carefully removed from the collection tube and placed into a new 2 ml collection tube. This was then centrifuged at room temperature for 1 minute at 8,000 g. The spin column was then transferred to a new 1.5 ml collection tube and 50 µl RNase-free water was added directly to the membrane. This was then centrifuged at room temperature for 1 minute at 8,000 g. 50 µl RNase free water was added to the samples a second time which were centrifuged at room temperature for 1 minute at 8,000 g. The resultant flow-through was then collected and transferred to 0.2 ml RNase free tubes and stored at -80°C.

2.2.10.2 RNA quantification and purity

Total RNA was quantified using a Qubit RNA Broad Range Assay (ThermoFisher, UK). Quant-iT™ Working Solution was prepared by diluting the Quant-iT™ reagent in Quant-iT™ buffer (1:200) and 190 µl of this working solution was distributed to each standard along with 10 µl standard solution. 199 µl Working Solution was added to each sample tube followed by 1 µl of sample. Standards and samples were vortexed for 2-3 seconds and incubated at room temperature for 2 minutes in the dark. Samples were read in duplicate using a Quibit™ fluorometer and the average reading was taken.

To check the purity of the RNA, the absorbance ratio at A260/280 was measured using a Nanophotometer® (Implen, USA). 1 µl RNase free dH₂O was used as a blank and 1 µl was used per sample measurement. RNA samples with A260/280 readings between 2.0 and 2.2 were considered ‘pure’.

2.2.10.3 Reverse transcription (RT) using a High-Capacity cDNA RT Kit

The 2x RT Master Mix was prepared in nuclease-free microcentrifuge tubes for the number of reactions required plus 10% overage according to the manufacturer’s protocol. The solutions were mixed gently but thoroughly and briefly centrifuged. The Master Mix was distributed to nuclease-free PCR tubes. Samples lysates were added to each aliquot of the RT Master Mix, gently mixed and then centrifuged briefly. The thermal cycler (GeneAmp® PCR System 9700; Applied Biosystems, USA) was set up to run using the following settings: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and then held at 4°C. Completed RT reactions were then stored at -20°C until required.

2.2.10.4 Measurement of gene expression by real-time PCR

The PCR Cocktail was prepared in a nuclease-free microcentrifuge tube (plus 10% overage) according to the manufacturer’s protocol; 5 µl Taqman Assay Master Mix, 0.5 µl Taqman primers
and 2 µl nuclease-free dH2O. 2.5 µl cDNA was then added to the PCR Cocktail in each well to make a final reaction volume of 10 µl. Samples were gently mixed with the tube closed, and briefly centrifuged. All samples were measured in triplicate including a non-template control in which cDNA was replaced with nuclease-free dH2O. The plate was sealed and the PCR reactions were run using the real-time PCR system, ViiA7™ (Applied Biosystems, USA) as per the following:
1 x cycle at 50°C for 2 minutes; 1 x cycle at 95°C for 2 minutes and then 40 x cycles at 95°C for 15 minutes and 60°C for 20 seconds.

2.2.10.5 Interpretation of results
Results of real-time PCR are given as C_q values, as calculated in section 2.2.6.5. In addition to the reference genes, three cell-specific genes were used as calibrator genes to normalise the data to account for changes in cell type number in disease. PDGFRβ and COL4A1 were used as pericyte-specific calibrator genes and PECAM1 was used as an endothelial-cell calibrator gene.

2.2.11 Sandwich enzyme-linked immunosorbent assay (ELISA)
EDNRA and EDNRB protein levels in human post-mortem tissue were measured using a human EDNRA or EDNRB commercial sandwich ELISA Kit (Antibodies.com).

2.2.11.1 Tissue homogenisation and SDS Buffer preparation
A 10% SDS lysis buffer solution without protease inhibitors was prepared in advance and stored at 4°C prior to use (see section 2.1.3 for solution constituents). Due to the short half-life of protease inhibitors, these were added at the time of use (1 mM PMSF (Sigma, USA): 1:100 diluted in ethanol; Aprotinin (Sigma, USA): 1.4 mg/ml) along with dH2O to make up to final 1% SDS working buffer. Frozen brain tissue from the parietal cortex was dissected (200 mg) and stored at -80°C. 7-10 zirconia/silica beads and 1 ml 1% SDS lysis buffer were added to each sample and were homogenised for x2 15 seconds at 6000 rpm in a Precellys 24 Homogeniser. Samples were then placed in a refrigerated centrifuge and centrifuged at 4°C for 20 minutes at 16,000 g. The resultant supernatant was transferred to a new homogenisation tube and stored at -80°C until use.

2.2.11.2 Sandwich ELISA procedure
The 96-well plate was pre-coated with capture antibody. Standards were added to the wells as per the manufacturer’s guidelines. 100 µl of sample (diluted to a working concentration determined by initially performing a serial dilution of brain homogenates) were then added to the sample wells. The plate was sealed and left to incubate at 37°C for 90 min. The seal was
removed and the wells were emptied by inverting the plate and patting onto tissue. The plate was then washed x2 with Wash Buffer and 100 µl Biotin-labelled antibody working solution was added into the wells. The plate was re-covered and sealed again and left to incubate at 37°C for 60 min. The cover was removed and the plate was washed x3 with Wash Buffer, letting the Wash Buffer sit in the wells for 1-2 min each time. 100 µl HRP-Streptavidin Conjugate Working Solution was added into each well, the plate was covered and sealed and left to incubate at 37°C for 30 min. The cover was removed and the plate was washed a further x5 with Wash Buffer, again letting the buffer sit in the well for 1-2 min each time. 90 µl TMB substrate was then added to each well and the plate was covered and sealed and left at 37°C for 10-20 min in the dark. 50 µl Stop Solution was added to each well to terminate the reaction (the colour will turn yellow). Optical density for absorbance was read at 450nm using a FLUOstar OPTIMA microplate reader. Wells were carried out in duplicate.
Chapter 3  Characterising contraction and proliferation of human foetal and adult brain-derived pericytes in response to EDN1

Note: Parts of this chapter are derived from an original research article that has undergone the peer review process and has been published (see Publication’s list (page ii) item 1). This work was undertaken by me during my period of study. Some of the contents may have been modified to ensure coherency and that integration of the manuscript into the thesis followed university submission guidelines.

3.1  Background

Pericytes are vascular mural cells that are particularly abundant within the brain. They are expressed within the capillary network but also reside on pre- and post-capillary vessels. Pericytes are critical components of the NVU and dynamically regulate CBF in response to local changes in metabolic demand(275, 278, 326). In stroke, pericytes die in rigor, clamping shut capillary lumina, contributing to ischaemic injury (even after re-opening the occluded vessel)(304, 327). Recent studies indicate that pericytes are damaged and degenerate in AD and VaD(252, 282, 328). This is thought to play a major role in BBB breakdown and cerebral hypoperfusion, and to impair clearance of Aβ in AD(247).

Brain ischaemia has long been recognized as the principal pathological process in VaD but recent studies have shown that cerebral hypoperfusion and BBB leakiness are early and substantial contributors to disease progression and cognitive decline in AD(328). It was previously shown that the enzymes responsible for synthesis of the vasoconstrictor peptide EDN1 are upregulated in AD(321, 322) and associated with elevated EDN1 level in the cerebral cortex. The level of EDN1 is correlated strongly with biochemical evidence of cerebral hypoperfusion in AD and with the level of Aβ, which was found to increase the production of EDN1 by neurons and endothelial cells in vitro(307). EDN1 induces contraction of SMCs, causing constriction of penetrating arteries and arterioles and reducing blood flow in the cerebral cortex and deep within the brain(329). EDN1 also causes contraction of pericytes contributing to constriction of capillaries in response to oligomeric Aβ, demonstrated in human and rodent tissue slices(275).

In vitro studies of pericyte function have been performed with bovine retinal pericytes(330) using semi-quantitative methods, such as the ‘wrinkling assay’ (pericyte contraction causes folds when the cells are grown on a silicone membrane(331)), and with fHBVP (ScienCell, USA)(326, 332). The foetal cells express pericyte markers such as PDGFRβ and NG2(333) and are negative
for endothelial markers and non-vascular markers including GFAP and IBA1(304). The cells respond to vasoactive metabolites and contract and migrate in culture(275, 326, 334). They have been shown to express α-SMA(242).

Foetal pericytes are responsive to multiple vasoactive peptides in the brain, and contract in response to EDN1 as previously shown using an electrical impedance assay(326). It remains unclear whether the pathophysiological responses of foetal brain pericytes reflect those in the adult brain. In this chapter, I have assessed EDN1-mediated contraction of fHBVP and aHBVP by use of an electrical impedance assay.

3.2 Hypothesis and Aims

3.2.1 Hypothesis

I explored the hypothesis that EDN1-mediated contraction and proliferation in human brain-derived pericytes were dependent on EDNRA expression and that impaired contractility of adult brain-derived pericytes is a potential contributor to cerebral hypoperfusion in AD.

3.2.2 Aims

The aims of this study were to characterise the EDN1-mediated contractile, relaxation and proliferative responses of fHBVP and aHBVP in vitro using a novel electrical impedance approach (xCELLigence).
3.3 Methods

3.3.1 Immunofluorescent labelling of pericytes

Expression of canonical pericyte markers, and EDNRA and EDNRB, were confirmed by immunofluorescence using the method detailed in section 2.2.2. Primary antibodies were applied as follows; EDNRA (1:500, Abcam UK, rabbit polyclonal), EDNRB (1:1000, Abcam UK, rabbit polyclonal), PDGFRβ (1:500, R&D Systems USA, biotinylated goat polyclonal) and α-SMA (1:100, Abcam UK, rabbit polyclonal). Secondary antibodies included donkey anti-rabbit IgG Alexa Fluor 488 (Invitrogen UK) for EDNRA, EDNRB and α-SMA and streptavidin Alexa Fluor 555 conjugate (Invitrogen, UK) for PDGFRβ.

To assess total receptor expression, cells were immunolabelled as outlined in section 2.2.2. However, to assess membrane-bound expression only, the cells were immunolabelled as normal but without the addition of methanol (membrane permeabilising agent) for 10 minutes. Six randomly selected images were acquired for cells with or without methanol using a fluorescence microscope (Nikon eclipse 80i). The images were analysed using Cell Profiler imaging software to identify individual cells and calculate the mean fluorescence intensity per cell in each image and this was used to calculate the average fluorescence intensity for each condition.

3.3.2 xCELLigence

Contraction, relaxation, and proliferation in response to EDN1 (Abcam, UK) of both fHBVP and aHBVP were characterised using xCELLigence, as outlined in section 2.2.3. Each condition was performed in duplicate wells and repeated in independent experiments. Using the xCELLigence data analysis software, the change in calculated cell index (slope) was used to reflect rates of contraction and relaxation (acute response < 30 minutes) and proliferation (chronic response 48 hours).

To assess the effect of receptor blockers on EDN1-mediated contraction, BQ123 (Tocris, UK) and BQ788 (Sigma-Aldrich, USA) were added in serum-free media 1 hour before the addition of EDN1.

3.3.3 BrdU proliferation assay

fHBVPs were seeded in a 96-well plate at 5,000 cells per well and left to adhere overnight. The cells were incubated with purified recombinant EDN1 (diluted in serum free medium) for 72 hours (n = 1). A BrdU incorporation assay (Abcam, UK) was used according to the manufacturer’s instructions to assess pericyte proliferation (as outlined in section 2.2.5).
3.4 Results

3.4.1 Pericytes isolated from foetal and adult human brain express pericyte markers

fHBVP and aHBVP expressed the canonical pericyte marker PDGFRβ (Figure 12A-B). Pericytes expressed α-SMA (Figure 12C-D) and both EDNRα (Figure 12E-F) and EDNRβ (Figure 12G-H).

![Figure 12. Representative immunofluorescence of canonical pericyte markers in fHBVP and aHBVP](image)

(A) fHBVP and aHBVP expressed PDGFRβ (Red; A, B), α-SMA (Green; C, D) and both EDNRα (Green; E, F) and EDNRβ (Green; G, H). All counterstained with a nuclear marker (DAPI – blue). Magnification 40×. Scale bar represents 100 μm.

3.4.2 EDN1 induced contraction and proliferation of fHBVP

Electrical impedance assays (xCELLigence) were used to assess pericyte contraction, relaxation, and proliferation in response to EDN1, as previously reported(326). fHBVP were treated with EDN1 at 1 pM, 10 pM, 0.1 nM, 1 nM, 0.01 μM, 0.1 μM, 1 μM. A vehicle control (pericyte medium only) was also run in parallel to provide a comparator baseline to generate the normalised contraction cell index curves (n = 12 independent experiments).

Immediately after addition of EDN1, pericyte contraction caused a rapid decrease in cell index (relative impedance), followed by a relaxation phase (~10 minutes) – a classic G-protein coupled receptor response – and subsequent proliferation, leading to a progressive rise in cell index over 48 - 72 hours (Figure 13).
The rate of contraction of fHBVPs increased with the concentration of EDN1 (0.01 μM – 1 μM) as illustrated in Figure 13A. The initial rapid decrease in cell index induced by EDN1 was significantly different from the cell index in the control well (medium only) after exposure to 0.01 μM (p = 0.0017), 0.1 μM (p < 0.0001) or 1 μM (p < 0.0001) EDN1 (n = 12) (Figure 13B). Contraction was followed by an increase in cell index (i.e., relaxation) as illustrated in Figure 13C. The rate of increase was related to the rate of contraction and was significantly higher in cells that had been exposed to 0.1 nM (p = 0.0005), 1 nM (p = 0.0032), 0.01 μM (p < 0.0001), 0.1 μM (p = 0.0001) or 1 μM (p < 0.0001) EDN1 (n = 12) (Figure 13D).

To monitor the effects of EDN1 on proliferation, the impedance was monitored for a further 48 - 72 hours following contraction (Figure 13E), during which impedance rose reflecting an increase in the number of cells covering the plate. The rate of increase was calculated and indicated that proliferation of fHBVP was significantly greater when exposed to 1 pM (p = 0.008), 0.1 nM (p = 0.005), 1 nM (p = 0.001), 0.01 μM (p = 0.001), 0.1 μM (p = 0.0011) or 1 μM (p = 0.0013) EDN1 than in untreated control cells (n = 12 independent experiments) (Figure 13F).
Figure 13. Contraction and proliferation of fHBVP in response to EDN1

(A) Representative electrical impedance measurements of the contractile response of fHBVP to EDN1. (B) The rate of contraction was significantly increased by addition of 0.01 μM (p = 0.0017), 0.1 μM (p < 0.0001) or 1 μM (p < 0.0001) EDN1 (n = 12). (C) Representative electrical impedance measurements of the relaxation response of fHBVP following contraction. (D) The increased rate of contraction was followed by more rapid relaxation in cells treated with 0.1 nM (p = 0.0005), 1 nM (p = 0.0032), 0.01 μM (p < 0.0001), 0.1 μM (p = 0.0001) or 1 μM (p < 0.0001) EDN1 (n = 12). (E) Representative electrical impedance measurements of the proliferative response of the fHBVP after EDN1. (F) Proliferation was significantly increased after the addition of 1 pM (p = 0.008), 0.1 nM (p = 0.005), 1 nM (p = 0.001), 0.01 μM (p = 0.001), 0.1 μM (p = 0.0011) or 1 μM (p = 0.0013) EDN1 (n = 12). The timeframe referred to in each graph is indicated by the red bracket. Arrows indicate time of EDN1 addition. Friedman test with Dunn’s multiple comparison post hoc test (B) and RM one-way ANOVA with Dunnett’s multiple comparison post hoc test (D, F) were used for comparisons. Data represent the mean values ± SEM.
3.4.3 **EDN1 mediates contraction of fHBVP via activation of EDNRA**

To determine the receptor specificity of the EDN1-mediated contractile response, fHBVP were pre-treated with either BQ123 or BQ788, antagonists of EDNRA and EDNRB, respectively \((n = 7)\). Receptor antagonists were added at 0.1 µM, 1 µM and 10 µM for 1 hour before EDN1 treatment (100 nM) across all wells. Two controls were also run in parallel, one with EDN1 only and one vehicle control to act as a baseline to generate the normalised contraction cell index curves.

Pre-treatment with BQ123, an EDNRA antagonist, inhibited EDN1-mediated pericyte contraction in a dose-dependent manner (Figure 14A). A significant reduction in the rate of contraction induced by EDN1 (100 nM) was observed in cells pre-treated with BQ123 at 1 µM \((p = 0.0284)\) or 10 µM \((p = 0.0002)\) \((n = 7)\) (Figure 14B). Pre-incubation with BQ788, an EDNRB receptor antagonist, did not significantly alter EDN1-induced pericyte contraction \((n = 7)\) (Figure 14C-D).
Figure 14. Contraction of fHBVP in response to EDN1 is mediated by EDNRA

(A) Representative electrical impedance measurements of the contractile response of fHBVP to EDN1 in the presence of BQ123, an EDNRA antagonist. (B) The rate of contraction was significantly reduced in cells to which 1 µM (p = 0.0284) or 10 µM (p = 0.0002) BQ123 had been added (n = 7). (C) Representative electrical impedance measurements of the contractile response of fHBVP in the presence of BQ788, an EDNRB antagonist. (D) There was no significant difference in the rate of contraction between BQ788-treated and untreated cells (n = 7). The timeframe referred to in each graph is indicated by the red bracket. Arrows indicate the timepoint of EDN1 addition. Kruskal-Wallis and Dunn’s multiple comparisons post hoc test were used for all comparisons. Data represent the mean values ± SEM.
The experiment was extended over a further 48 hours to monitor the effects of EDNRA and EDNRB antagonism on EDN1-induced proliferation (n = 7). Neither pre-incubation with BQ123 or BQ788 significantly altered EDN1-induced pericyte proliferation (n = 7) (Figure 15A-D).

**Figure 15. Proliferative response of fHBVP in response to EDN1 in the presence of EDNRA and EDNRB antagonists**

(A) Representative electrical impedance measurements of the proliferative response of fHBVP to EDN1 (100 nM) in the presence of BQ123, an EDNRA antagonist (n = 1). (B) There was no significant difference in the rate of proliferation between BQ123-treated and untreated cells (n = 7). (C) Representative electrical impedance measurements of the contractile response of fHBVP in the presence of BQ788, an EDNRB antagonist (n = 1). (D) There was no significant difference in the rate of proliferation between BQ788-treated and untreated cells (n = 7). Timeframe referred to in each graph indicated by red bracket. Arrow indicates timepoint of EDN1 addition. RM one-way ANOVA with Dunnett’s multiple comparison post hoc test (B) and Friedman test with Dunn’s multiple comparison post hoc test (D) were used for comparisons. Data represent the mean values ± SEM.
3.4.4 aHBVP contract in response to EDN1 but do not proliferate

aHBVP were treated with EDN1 at 1 pM, 10 pM, 0.1 nM, 1 nM, 0.01 µM, 0.1 µM, 1 µM and a vehicle control (pericyte medium only; n = 4). The number of independent replicates was lower due to difficulties in maintaining healthy cultures of adult pericytes.

EDN1 induced contraction of aHBVP. The rate of initial decline of cell index (i.e., contraction) was dose-related (1 pM - 1 µM), as illustrated in Figure 16A. The rate of contraction was significantly higher in adult cells treated with 0.01 µM (p = 0.0438), 0.1 µM (p = 0.0001) or 1 µM (p < 0.0001) EDN1 than in untreated cells (n = 4) (Figure 16B). The initial contraction was followed by a slower increase in cell index (i.e., relaxation), as shown in Figure 16C; the increase in cell index was most rapid after 0.1 µM (p = 0.0043) and 1 µM (p = 0.0203) EDN1 exposure (n = 4) (Figure 16D).

Impedance was monitored for a further 48 hours to assess the effect of EDN1 on adult pericyte proliferation. The cell index increased slowly (Figure 16E) and was not affected by exposure to EDN1 (n = 4) (Figure 16F).
Figure 16. Contraction and proliferation of aHBVP is mediated by EDN1

(A) Representative electrical impedance measurements of the contractile response of aHBVP to EDN1. (B) The rate of contraction was significantly higher after adding 0.01 µM (p = 0.0438), 0.1 µM (p = 0.0001) or 1 µM (p < 0.0001) EDN1 (n = 4). (C) Representative electrical impedance measurements of the relaxation response of aHBVP following contraction. (D) The rate of subsequent relaxation was significantly higher in cells to which 0.1 µM (p = 0.0043) and 1 µM (p = 0.0203) EDN1 had been added (n = 4). (E) Representative electrical impedance measurements of the proliferative response of the aHBVP to EDN1. (F) No significant difference in rate of proliferation between EDN1-treated and control cells was found (n = 4). The timeframe referred to in each graph is indicated by the red bracket. Arrows indicate the timepoint of EDN1 addition. One-way ANOVA and Dunnett’s multiple comparison post hoc test (B), Kruskal-Wallis and Dunn’s multiple comparison post hoc test (D) and RM one-way ANOVA with Dunnett’s multiple comparison post hoc test (F) were used for comparison. Data represent the mean ± SEM.
3.4.5 **EDN1 mediates contraction of aHBVP via activation of EDNRA**

aHBVP were pre-treated with either BQ123 or BQ788 before EDN1 addition to determine if contraction was mediated through EDNRA or EDNRB as observed in fHBVP. Cells were pre-treated with receptor antagonists which were added at 0.1 µM, 1 µM and 10 µM 1 hour before EDN1 treatment (100 nM) across all wells. Two controls were run in parallel, one with EDN1 only and one vehicle control to as a baseline to generate normalised contraction cell index curves (n = 7).

Pre-treatment with BQ123 inhibited EDN1-mediated contraction of aHBVP (Figure 17A). A significant reduction in the rate of contraction induced by EDN1 (100 nM) was seen in pericytes pre-treated with 10 µM ($p = 0.0214$) BQ123 compared to cells treated with EDN1 alone (n = 7) (Figure 17B). Pre-treatment with BQ788 did not significantly influence EDN1-induced adult pericyte contraction (Figure 17C-D).
Figure 17. Contraction of aHBVP in response to EDN1 is mediated by EDNRA

(A) Representative electrical impedance measurements of the contractile response of aHBVP to EDN1 in the presence of BQ123, an EDNRA antagonist. The rate of contraction was significantly reduced in cells to which 10 µM (p = 0.0214) BQ123 had been added (n = 7). (C) Representative electrical impedance measurements of the contractile response of aHBVP in the presence of BQ788, an EDNRB antagonist. (D) There was no significant difference in the rate of contraction between BQ788-treated and untreated cells (n = 7). The timeframe referred to in each graph is indicated by the red bracket. Arrows indicate the timepoint of EDN1 addition. Friedman test with Dunn’s multiple comparison post hoc test (B) and RM one-way ANOVA with Dunnett’s multiple comparison post hoc test (D) were used for comparisons. Data represent the mean values ± SEM.
The experiment was extended for a further 48 hours to monitor the effects of EDNRA and EDNRB antagonism on EDN1-induced proliferation (n = 7). Neither pre-incubation with BQ123 or BQ788 significantly altered the pericyte response to EDN1 (n = 7) (Figure 18A-D).

Figure 18. Proliferative response of aHBVP in response to EDN1 in the presence of EDNRA and EDNRB antagonists

(A) Representative electrical impedance measurements of the proliferative response of aHBVP to EDN1 (100 nM) in the presence of BQ123, an EDNRA antagonist (n = 1). (B) There was no significant difference in the rate of proliferation between BQ123-treated and untreated cells (n = 7). (C) Representative electrical impedance measurements of the contractile response of aHBVP in the presence of BQ788, an EDNRB antagonist (n = 1). (D) There was no significant difference in the rate of proliferation between BQ788-treated and untreated cells (n = 7). Timeframe referred to in each graph indicated by red bracket. Arrow indicates timepoint of EDN1 addition. Friedman test and Dunn’s multiple comparisons post hoc test (B) and Kruskal-Wallis and Dunn’s multiple comparison post hoc test were used for comparisons. Data represent the mean values ± SEM.
3.4.6 Endothelin receptor expression is reduced in aHBVP

To determine whether adult pericytes were less sensitive than foetal pericytes to EDN1 because of altered receptor expression, fHBVP and aHBVP were immunolabelled for EDNRA, EDNRB and \( \alpha \)-SMA. Membrane-bound \( (p = 0.0017) \) and total \( (p < 0.0001) \) EDNRA expression was significantly lower in aHBVP than in fHBVP \( (n = 3) \) (Figure 19A-B). Membrane bound EDNRB expression was not significantly different between fHBVP and aHBVP (Figure 19C); however, total EDNRB expression was significantly lower in aHBVP \( (p = 0.0355) \) \( (n = 3) \) (Figure 19D). Membrane bound \( (p = 0.0002) \) and total \( (p = 0.0010) \) \( \alpha \)-SMA expression was also significantly lower in aHBVP than in fHBVP \( (n = 3) \) (Figure 19E-F).
Figure 19. Change in membrane-bound and total EDNRA, EDNRB and α-SMA in fHBVP and aHBVP

(A) There was significantly less membrane-bound EDNRA labelling in aHBVP (p = 0.0017) than in fHBVP (n = 3). (B) There was significantly less total EDNRA labelling in aHBVP (p < 0.0001) than in fHBVP (n = 3). (C) No significant difference in membrane-bound EDNRA labelling between fHBVP and aHBVP was found (n = 3). (D) There was significantly less total EDNRB labelling in aHBVP (p = 0.0355) than in fHBVP (n = 3). (E) There was significantly less membrane-bound α-SMA labelling in aHBVP (p = 0.0002) than in fHBVP (n = 3). (F) There was also significantly less total α-SMA expression in aHBVP (p = 0.0010) than in fHBVP (n = 3). A two-tailed unpaired t test was used for all comparisons. Data represent the mean values ± SEM. Representative immunofluorescence images are included for each graph. Scale bars represent 50 µM.
3.5 Discussion

In this chapter, I have used an electrical impedance assay (xCELLigence) to characterize the contractile and proliferative responses of fHBVP and aHBVP to EDN1. EDN1-induced contraction of fHBVP and aHBVP was mediated specifically by activation of EDNRA. The contractile and relaxation responses were stronger in the foetal than adult cells, the former proliferated after EDN1-induced contraction whereas the latter did not. After measuring and comparing the levels of membrane-bound and total endothelin receptor expression between fHBVP and aHBVP, I have shown aHBVP express significantly less EDNRA and α-SMA which could account for the decreased sensitivity and relaxation response of aHBVP to EDN1.

3.5.1 EDN1 mediates contraction of human foetal pericytes via EDNRA

I have optimised the use of xCELLigence, to measure contraction, relaxation, and proliferation of human brain pericytes. This technique enabled changes in contraction and proliferation to be measured in real-time without the use of fluorescent labels. Previous studies have demonstrated pericyte contraction in response to EDN1 by directly measuring the change in cell size using light microscopy or dyes, or using ‘wrinkling’ assays, but as is shown by the use of xCELLigence, pericytes are highly sensitive to changes in their immediate environment (such as slight morphological and structural changes to the physical addition of serum-free media) which previous studies failed to take into account(335, 336).

Pericytes respond to vasoactive stimuli; ATP(337) and EDN1(338) induce pericyte contraction whereas adenosine and nitric oxide cause pericyte relaxation(326). In human and rat cortical slices, noradrenaline constricts, and glutamate dilates capillaries in proximity to pericyte cell bodies(275, 304, 339). EDN1 was previously reported to cause pericyte contraction of bovine retinal pericytes(338), associated with the release of cytosolic Ca^{2+} and reorganization of actin filaments(336). More recently, EDN1 was shown to induce strong pericyte-mediated contraction reducing the lumina of capillaries in rat cortical slices – an effect mediated by the EDNRA(275, 326). Neuhaus et al., demonstrated the utility of xCELLigence to first measure contraction, relaxation, and proliferation of fHBVP in response to EDN1 and various vascular stimuli(326). I have confirmed that EDN1 induces contraction of fHBVP, across a similar dose range to that previously reported (0.1 nM – 1 μM), reflecting the normal concentration of EDN1 in human plasma (3.55 ± 1.78 pg/ml)(340). The contractile response was dose-dependent and was typical of a classical G-protein coupled response(341). Subsequent relaxation was also dose
dependent and was related to the extent of contraction; the greater the contractile response, the greater the relaxation to return to resting state.

This study has also demonstrated that EDNRA is responsible for EDN1-mediated contraction of fHBVP. This is in keeping with existing literature that suggests EDNRA mediates vasoconstriction(342) whereas EDNRB activation has vasodilatory effects(320). Interestingly, the rate of proliferation was not significantly inhibited by EDNRA antagonism. This contradicts findings by Neuhaus who demonstrated a significant reduction in the rate of proliferation and increased overall doubling time upon endothelin receptor antagonism(326). Other studies also show that EDN1 induces pericyte proliferation via EDNRA(343). The current data suggest that whilst EDNRA mediates contraction, EDN1-mediated proliferation is facilitated by another mechanism/receptor (although experiments using repeated exposure to EDN1 over the longer time course would need to be performed to confirm this). The reason for disparity in these findings is unclear.

Studies have shown that PDGF-BB and its receptor PDGFRβ are responsible for proliferation in various experimental and disease settings(344, 345) and that EDN1 can regulate proliferation in synergy with PDGF-BB in SMCs(346) and potentiate these effects up to six-fold(347). Overall, this raises the possibility of an interaction between EDN1, PDGF-BB and PDGFRβ, responsible for pericyte proliferation; this potential interaction is worth exploring in future studies.

3.5.2 EDN1 mediates contraction of human adult pericytes via EDNRA

I have extended previous findings and used xCELLigence to characterise the contractile and proliferative responses of aHBVP to EDN1. My findings demonstrate contraction of adult pericytes in response to EDN1. Like fHBVP, aHBVP contraction resulted in an initial rapid decrease in cell index and contraction was dose dependent. These cells are of adult origin and are therefore more representative of age-related disease such as AD.

Compared to foetal pericytes, adult pericytes appear less responsive to EDN1. The minimum concentration of EDN1 needed to elicit contraction in aHBVP was 0.1 μM – 10 times greater than needed for contraction of fHBVP. Subsequent two-way ANOVA analysis (summary statistics see appendix 8.1.8) revealed a significant reduction in rate of contraction and relaxation in aHBVP compared to fHBVP, some cells remaining partially contracted, as demonstrated by the slower increase in cell index following contraction compared to that for fHBVP. A previous study in aged VSMC also demonstrated impaired contraction and relaxation compared to younger cells. The authors demonstrated impaired α1 adrenergic receptor-mediated contractile
responses in VSMC to agonists including angiotensin II and phenylephrine. Subsequent 3D imaging of VSMC in collagen gels showed a reduced contractile force generated by aged cells due to reduced α-SMA expression (a 40% decrease in actin fibres in aged rats was observed(348), similarly shown in this study (see section 3.5.3)).

The impaired contractile and relaxation responses of aHBVP to EDN1 is interesting and could potentially negatively affect neurovascular coupling and contribute to cerebral hypoperfusion in normal ageing. The distinction most likely reflects differences in sensitivity between adult and foetal pericyte populations – adult pericytes being less sensitive than foetal. This phenomenon is seen in the case of GPCR desensitisation after prolonged ligand binding via agonist-induced phosphorylation. Adult cell populations display affected receptor internalisation, decreased receptor coupling efficiency and decrease ligand affinity compared to cells from younger animals(349, 350).

Unlike fHBVP, which proliferated in response to EDN1 as reported previously(326), aHBVP did not. It may be due to desensitisation to external agonists as previously discussed combined with the already diminished proliferative capacity that these ‘aged’ cells exhibit. Cells have a limited number of times they can divide; a phenomenon termed the Hayflick limit(351). Cellular senescence is a process by which cells stop dividing once they have reached their replicative capacity or Hayflick limit, and has a role in development and ageing, and protection against cancer(352). Senescent cells undergo phenotypic and physiological alterations which include changes in receptor expression and sensitivity to agonist binding(353). Cells at a higher passage number have undergone more replications, potentially resulting in senescence. This means their proliferative capacity in the presence of EDN1 may be diminished. Whether this reflects a genuine physiological difference between pericytes obtained from foetal and adult brains or is specific to this commercial source of pericytes (Cell-Systems), remains to be determined. Further studies to characterize the morphology and physiology of foetal and adult brain-derived pericytes and their anatomical subtypes should provide important insights into age- and disease-related pericyte dysfunction.

Like fHBVP, this study also demonstrates novel EDNRA specificity in the adult pericyte population in mediating contraction in response to EDN1. The fact that these adult cells also contract via EDN1 binding to EDNRA, further substantiates the hypothesis that hyper-contraction in these pericytes by increased EDN1 contributes to cerebral hypoperfusion in AD. Similarly, this study found endothelin receptor antagonism did not alter the proliferative responses compared to cells treated with EDN1 alone. As previously discussed, this is likely due
to alternative receptor activation that could not be identified through endothelin receptor antagonism.

3.5.3 aHBVP express less endothelin receptor and α-SMA

I show that aHBVP express significantly less EDNRA than fHBVP cells cultured under the same conditions. As I also demonstrated EDNRA specificity in facilitating EDN1-mediated contraction of human brain pericytes, the current data suggest that reduced EDNRA expression in adult pericytes could potentially contribute to their reduced responsiveness to EDN1-mediated contraction (and delayed relaxation to an uncontracted state).

aHBVP also expressed significantly less α-SMA immunolabelling. α-SMA has been suggested to play a role in the production of contractile force(354) as demonstrated in wound healing studies, and Hinz et al. demonstrated that enhanced contractile activity of fibroblasts correlated with increased α-SMA expression(355). With α-SMA playing such an integral role in cell contractility, the data herein suggests that a lower sensitivity of adult pericytes to EDN1 in mediating contraction compared to foetal pericytes partly reflects reduced contractile protein expression.

3.5.4 Summary of key points

Foetal and adult pericytes contract in response to physiological levels of EDN1 and subsequently relax. The response is mediated by EDNRA, although subsequent proliferation of fHBVPs is not inhibited by EDNRA receptor antagonism. Interestingly, adult-derived pericytes were less responsive than foetal pericytes to EDN1 (up to x100 fold higher EDN1 concentration was required to elicit contraction). Following EDN1-mediated contraction, adult cells displayed significantly reduced rates of contraction and relaxation, taking longer to relax back to baseline. Adult cells also failed to proliferate in response to EDN1. A reduction in EDNRA expression and/or α-SMA expression may be responsible for their reduced capacity to respond to EDN1.
Chapter 4  Investigating the effects of Aβ on EDN1-mediated contraction of HBVP

Note: Parts of this chapter are derived from an original research article that has undergone the peer review process and has been published (see Publication’s list (page ii) item 1). This work was undertaken by me during my period of study. Some of the contents may have been modified to ensure coherency and that integration of the manuscript into the thesis followed university submission guidelines.

4.1  Background

In the previous chapter, I characterised EDN1-mediated contractile responses of fHBVP and aHBVP using a novel in vitro electrical impedance assay. This next chapter describes the characterisation of these contractile responses under conditions that reflect those in the AD brain.

In rodent models of AD, vascular dysfunction including reduced blood flow and BBB leakiness precede the onset of cognitive decline and disease pathology(195, 199, 356). A reduction in CBF correlates with increased Aβ deposition as well as memory impairment(357). Ischemia and hypoxia have been shown to upregulate BACE1, the enzyme responsible for producing Aβ which leads to subsequent neurodegeneration and cognitive decline(210, 358). Damage to the cerebral vasculature, specifically the BBB, is also likely to impair the clearance of Aβ (and other dementia-related proteins including tau and TDP-43) out of the brain(359). Aβ also has vasoconstrictor properties acting on arteries(216) and pericytes and can have direct toxic effects on the cerebral vasculature at high concentrations(360). Together, these studies indicate a bi-directional relationship between Aβ and vascular dysfunction in AD.

Transgenic mice overexpressing APP and Aβ have been shown to exhibit a profound reduction in the CBF response to endothelium-dependent vasodilators or neural activation(195, 356, 361, 362). These effects are also observed after application of synthetic Aβ to the cerebral cortex of wild-type mice and are counteracted by free radical scavengers(363). In contrast, Sharp et al., using 2D-optical imaging spectroscopy, found no difference in CBF observing AD-mice and wild-type controls. However, subsequent acute electrophysiology revealed previously unidentified CBF deficits in the same mice. Their work highlighted that CBF changes were observed only when using a more invasive method of CBF measurement and that the change in CBF was likely due to the inability of the mouse to cope with an additional insult. This is something previous
studies fail to consider(364). Although previous studies have investigated the constrictor effect of Aβ on isolated arteries, those studies had limitations related to the use of pharmacologically pre-constricted isolated vessels(365, 366). Investigations into the vascular effects of Aβ have primarily focused on large arterioles(216, 367); however, most of the vascular resistance in the brain occurs at the capillary level(274).

Post-mortem tissue studies by Palmer and colleagues revealed an increase in ECE-2 (an enzyme responsible for EDN1 production) and EDN1 in AD in the temporal cortex as well as an upregulation in their expression in response to Aβ1-42 in vitro(322). The same authors later found a significant increase in ECE-1 activity and EDN1 level in AD post-mortem vessels (and this was specific to AD as no change was seen with respect to VaD), which was increased in vitro following Aβ1-40 exposure(321). The same group has demonstrated significant correlations between elevated EDN1 and biochemical markers of pathological hypoperfusion: decline in MAG:PLP1 (a marker of chronic reduction in tissue oxygenation)(206), as well as elevated VEGF-A(308), a marker of recent reduction in oxygenation) suggesting possible Aβ-mediated vasoconstriction of perforating arterioles.

A recent study confirmed that capillaries were constricted in proximity to Aβ plaques in human biopsy brain tissue. Mechanistically, ROS production, in response to oligomeric Aβ, mediated EDN1-induced pericyte contraction and consequent narrowing of capillaries in rodent brain cortical slices. This study indicates that Aβ induced EDN1-mediated vasoconstriction is likely to be a major contributor to cerebral hypoperfusion in AD(275). Therefore, in this chapter, I have investigated whether pre-exposure of pericytes to Aβ affected EDN1-induced contraction in vitro as assessed using electrical impedance assays.

4.2 Hypothesis and Aims

4.2.1 Hypothesis

The hypothesis tested in this chapter was that exogenously applied Aβ would exacerbate the contractile response of pericytes to EDN1.

4.2.2 Aims

The aim of this study was to characterise the contraction and relaxation of HBVP in response to EDN1 in vitro after pre-exposure to Aβ peptides using electrical impedance assays (xCELLigence).
4.3  Methods

4.3.1  xCELLigence

Contraction, relaxation, and proliferation of fHBVP were characterised using xCELLigence which was performed as outlined in section 2.2.3. Aβ peptides (rPeptide, USA, diluted in 35% acetonitrile as a stock and diluted in serum-free medium) (0.001µM – 10 µM) were added 1 or 24 hours prior to EDN1 application. Experiments were carried out in duplicate wells. Built in software was used to calculate the change in cell index (slope) to measure rates of contraction and relaxation (acute response < 30 minutes) and subsequent proliferation of the cells (chronic response 48 hours).

4.3.2  Cell viability assay

Cell viability was assessed as outlined in section 2.2.4. fHBVP and aHBVP were seeded in black clear bottomed 96-well plates at 5,000 cells per well and left to settle overnight. Pericytes were then incubated with recombinant Aβ peptides (rPeptide, USA, diluted in 35% acetonitrile as a stock and diluted in serum-free medium) for 24 hours. Cell toxicity was assessed by measurement of uptake of ethidium dye, in the Invitrogen live/dead mammalian cell assay (Invitrogen, UK), according to the manufacturer’s guidelines (n = 2). The percentage of cells that had died was calculated with reference to the untreated control wells.
4.4 Results

I studied the effects of pre-exposure of Aβ peptides in fHVBPs only, as aHVBPs were not robust enough to provide reliable data. During experiments performed in Chapter 3, it was noticed that adult pericytes tended to proliferate more slowly. It was difficult to obtain a sufficient amount of healthy cells to perform robust experiments and using adult cells was more time-consuming and often more temperamental requiring additional repeats. Although a slower growth rate may be of biological relevance, it also made them impractical to use for these types of assays which required exposure to additional cellular insults. Given the slow proliferation rate and the large number of cells required to perform these experiments it was decided to focus on foetal pericytes only.

The concentration range of soluble Aβ tested (0.1-10 µM) broadly mirrors previous studies (275, 368). These concentrations of Aβ are considered to be supra-physiological.

4.4.1 The effect of short-term Aβ exposure on EDN1-mediated contraction of fHBVP

To investigate the acute effects of exogenously applied Aβ peptides on EDN1-induced contraction of fHBVP, cells were pre-incubated with Aβ1-40 or Aβ1-42 (0.1 µM, 1 µM or 10 µM) 1 hour before the addition of EDN1 (100 nM). Two controls were included: untreated (100 nM EDN1 only) and a vehicle control to act as a baseline to generate the normalised contraction cell index curves (n = 6 independent experiments).

Neither contraction nor relaxation was significantly altered by 1-hour exposure to Aβ1-40 or Aβ1-42 (Figure 20A-H).
Figure 20. Influence of Aβ for 1 hour on fHBVP contraction

(A) Representative electrical impedance measurements of the contractile response of fHBVP to EDN1 (100 nM) after 1 h incubation with Aβ_1-40 (n = 1). (B) There was no significant difference between the rate of contraction of EDN1-treated cells following pre-exposure to Aβ_1-40 (n = 7). (C) Representative electrical impedance measurements of the relaxation response of fHBVP following contraction (n = 1). (D) There was also no significant difference in rate of subsequent relaxation (n = 7). The timeframe referred to in each graph is indicated by the red bracket. Arrows indicate the timepoint of EDN1 addition.

RM one-way ANOVA and Dunnett’s multiple comparisons post hoc test were used for all comparisons. Data represent the mean values ± SEM.
4.4.2 Long-term exposure of Aβ alters the contractile response of fHBVP

I next determined if longer exposure to Aβ peptides for 24 hours impacted fHBVP contractility. Aβ₁₄₀ or Aβ₁₄₂ at 0.1 µM, 1 µM or 10 µM was added to the cell culture medium 24 hours before the addition of EDN1 (100 nM). Two controls were included: untreated (100 nM EDN1 only) and a vehicle control to act as a baseline to generate the normalised contraction cell index curves (n = 6 independent experiments).

Pre-treatment with 1 µM Aβ₁₄₀ for 24 hours significantly impaired EDN1-induced pericyte contraction (p = 0.0255; Figure 21A-B). Subsequent relaxation was also significantly altered in cells treated with 1 µM Aβ₁₄₀ (p = 0.0237; Figure 21C-D). Pre-treatment with 1 µM (p = 0.0216) and 10 µM (p = 0.0103) Aβ₁₄₂ for 24 hours significantly impaired EDN1-induced pericyte contraction (Figure 21E-F). Subsequent relaxation was also significantly altered in cells treated with 1 µM (p = 0.0113) and 10 µM (p = 0.0241) Aβ₁₄₂ (Figure 21E-H).
Figure 21. Influence of Aβ for 24 hours on fHBVP contraction

(A) Representative electrical impedance measurements of the contractile response of fHBVP to EDN1 (100 nM) after 24 hour incubation with Aβ1-40 (n = 1). (B) There was a significant difference between the rate of contraction of untreated cells and those exposed to 1 µM (p = 0.0255) Aβ1-40 (n = 9). (C) Representative electrical impedance measurements of the relaxation response of fHBVP following contraction (n = 1). (D) There was a significant difference between the rate of relaxation of untreated cells and those exposed to 1 µM (p = 0.0237) Aβ1-40 (n = 9). (E) Representative electrical impedance measurements of the contractile response of fHBVP to EDN1 (100 nM) after 24 hour treatment with Aβ1-42 (n = 1). (F) There was a significant difference between the rate of contraction of untreated cells and those exposed to 1 µM (p = 0.0216) and 10 µM (p = 0.0103) Aβ1-42 (n = 9). (G) Representative electrical impedance measurements of the relaxation response of fHBVP following contraction (n = 1). (H) There was a significant difference in rate of relaxation in cells treated with 1 µM (p = 0.0113) and 10 µM (p = 0.0241) Aβ1-42 (n = 9). The timeframe referred to in each graph is indicated by the red bracket. Arrows indicate the timepoint of EDN1 addition. Kruskal-Wallis and Dunn’s multiple comparisons post hoc test were used for all comparisons. Data represent the mean values ± SEM.
4.4.3 Assessing the effect of lower concentrations of Aβ on EDN1-mediated contraction

I next sought to determine if lower concentrations of Aβ peptide, which more closely resemble physiological concentrations found in AD, affected fHBVP contractility. These data are presented separately as they were generated using individually normalised baseline cell index values at a different timepoint.

Aβ_{1-40} or Aβ_{1-42} at 0.001 µM, or 0.01 µM was added to the cell culture medium 1 hour before the addition of EDN1 (100 nM). Two controls were included: untreated (100 nM EDN1 only) and a vehicle control to act as a baseline to generate the normalised contraction cell index curves (n = 9 independent experiments).

Neither contraction nor relaxation was significantly altered by 1-hour exposure to Aβ_{1-40} or Aβ_{1-42} (Figure 22A-H).
Figure 22. Influence of smaller concentrations of Aβ for 1 hour on fHBVP contraction

(A) Representative electrical impedance measurements of the contractile response of fHBVP to EDN1 (100 nM) after 1 hour incubation with Aβ_{1-40} (n = 1). (B) There was no significant difference between the rate of contraction of untreated cells and those exposed to Aβ_{1-40} (n = 9). (C) Representative electrical impedance measurements of the relaxation response of fHBVP following contraction (n = 1). (D) There was no significant difference between the rate of relaxation of untreated cells and those exposed Aβ_{1-40} (n = 9). (E) Representative electrical impedance measurements of the contractile response of fHBVP to EDN1 (100 nM) after 1 hour treatment with Aβ_{1-42} (n = 1). (F) There was no significant difference between the rate of contraction of untreated cells and those exposed Aβ_{1-42} (n = 9). (G) Representative electrical impedance measurements of the relaxation response of fHBVP following contraction (n = 1). (H) There was also no significant difference in rate of subsequent relaxation (n = 9). The timeframe referred to in each graph is indicated by the red bracket. Arrows indicate the timepoint of EDN1 addition. RM one-way ANOVA and Dunnett’s multiple comparisons post hoc test (B, D, H) and Friedman test with Dunn’s multiple comparison post hoc test (F) were used for all comparisons. Data represent the mean values ± SEM.
To investigate the long-term effects of exposure to these concentrations, Aβ\textsubscript{1-40} or Aβ\textsubscript{1-42} at 0.001 μM or 0.01 μM were added to the cell culture medium 24 hours before the addition of EDN1 (100 nM). Two controls were included: untreated (100 nM EDN1 only) and a vehicle control to act as a baseline to generate the normalised contraction cell index curves (n = 9 independent experiments).

Neither contraction nor relaxation was significantly altered by 24-hour exposure to Aβ\textsubscript{1-40} or Aβ\textsubscript{1-42} (Figure 23A-H).
Figure 23. Influence of smaller concentrations of Aβ for 24 hours on fHBVP contraction

(A) Representative electrical impedance measurements of the contractile response of fHBVP to EDN1 (100 nM) after 24 hour incubation with Aβ1-40 (n = 1). (B) There was no significant difference between the rate of contraction of untreated cells and those exposed to Aβ1-40 (n = 9). (C) Representative electrical impedance measurements of the relaxation response of fHBVP following contraction (n = 1). (D) There was no significant difference between the rate of relaxation of untreated cells and those exposed to Aβ1-40 (n = 9). (E) Representative electrical impedance measurements of the contractile response of these fHBVP to EDN1 (100 nM) after 24 hour treatment with Aβ1-42 (n = 1). (F) There was no significant difference between the rate of contraction of untreated cells and those exposed to Aβ1-42 (n = 9). (G) Representative electrical impedance measurements of the relaxation response of fHBVP following contraction (n = 1). (H) There was also no significant difference in rate of subsequent relaxation (n = 9). The timeframe referred to in each graph is indicated by the red bracket. Arrows indicate the timepoint of EDN1 addition. RM one-way ANOVA and Dunnett’s multiple comparisons post hoc test (B), Friedman test and Dunn’s multiple comparison post-hoc test (D, F) and Kruskal-Wallis and Dunn’s multiple comparison post hoc test (H) were used for comparisons. Data represent the mean values ± SEM.
4.4.4 Pericyte viability after exposure to Aβ peptides

To determine whether impaired EDN1-mediated pericyte contractile responses to Aβ at higher concentrations were due to toxicity, a cell viability assay was performed (by Dr Scott Miners) (Figure 24). Exposure to Aβ1-40 and Aβ1-42 for 24 hours at concentrations above 1µM caused pericyte death. Aβ1-40 and Aβ1-42 1µM caused ~20% pericyte death whereas a concentration of 10 µM caused between 50-60% cell death in fHVBPs.

![Figure 24](image)

Figure 24. fHBVP and aHBVP viability after exposure to Aβ peptides

(A) fHBVP and (B) aHBVP cell death after pre-exposure to Aβ1-40 and Aβ1-42. Exposure to Aβ1-40 and Aβ1-42 for 24 hours at concentrations above 1 µM caused pericyte death, indicated by the incorporation of ethidium-1 dye. Pericyte death was minimal at Aβ1-40 and Aβ1-42 levels below 1 µM. Data represent the mean values ± SD. Data courtesy of Dr Scott Miners, Dementia Research group, University of Bristol.
4.5 Discussion

In this study I have used electrical impedance assays (xCELLigence) to investigate the effects of acute and chronic exposure of Aβ peptide on the contractile responses of fHBVP. I have shown that whilst short-term exposure to Aβ has no effect, exposure to high concentrations of Aβ₁₋₄₀ impairs contraction.

4.5.1 Chronic Aβ exposure alters EDN1-mediated contraction in fHBVP

Soluble Aβ peptides, consisting of monomeric and oligomeric species, cause a slow constriction of brain capillaries, mediated by pericyte contraction, reducing the diameter of the vessel lumen by up to 25% in human and rat cortical sections(275). Transgenic rats infused with Aβ₁₋₄₀ into the lateral ventricles showed an increase in EDNRA expression in the cerebral cortex, hippocampus, and brain stem(369). I therefore investigated whether pre-exposure to soluble Aβ had an impact on EDN1-induced contraction of fHBVP and aHBVP in vitro.

Short-term exposure had no effect. Higher concentrations of Aβ (in the micromolar range, like those tested by Nortley(275)) for 24 hours impaired EDN1-mediated pericyte contraction. Pericytes are known to internalise Aβ through receptor-mediated uptake via LRP1(102, 370). Through uptake, Aβ may disrupt intracellular calcium signalling or directly interact with contractile proteins and the contractile machinery and lead to impaired cell contraction. Previous studies have demonstrated that oligomeric Aβ disrupted calcium homeostasis in neurons and cerebral endothelial cells via disrupted NMDAR-mediated calcium influx(371, 372). Future work investigating the effects of Aβ on calcium signalling in human brain pericytes would be of interest. The affected contractile response could also be a compensatory mechanism exhibited by pericytes. High amounts of Aβ, restrict blood flow within the brain seen in cerebral vascular diseases including CAA(91, 373). The high concentrations of Aβ may promote an inhibitory effect in the pericytes ability to elicit a contractile response to prevent further vasoconstriction and occlusion of the capillary, in the effort to maintain and restore CBF. Lastly, Aβ could affect endothelin receptor expression, decreasing the availability of functional receptors for subsequent EDN1 agonism. This will be explored in the subsequent chapter.

The rate of relaxation was also impaired upon exposure to Aβ, suggesting that the pericytes remain partially contracted. This is supported by previous studies that have demonstrated incomplete relaxation of pericytes and dilation of capillaries in disease following reperfusion(304, 339, 374). In one study, live cell imaging of cerebral cortical slices exposed to simulated ischaemia revealed a rapidly decreasing number of pericytes and death in rigor
following constriction of capillaries (304). In another study, mouse brain pericytes remained contracted after middle cerebral artery reperfusion following previous occlusion (339).

It must be noted that the effect of Aβ at high concentrations may have been partly because of cell toxicity as the data shows up to 60% pericyte degeneration at Aβ concentrations similar to those that impaired contraction (> 1 μm). Previous studies found that exposure to 5-25 μM Aβ_{1-40} over 3 to 7 days caused degenerative changes (252, 360) whereas 1.4 μM for 3 hours did not cause pericyte death in rat cortical slices (275). No evidence of pericyte degeneration at concentrations of Aβ_{1-40} or Aβ_{1-42} below 1 μM over a 24-hour period was found in this study.

Additional two-way ANOVA analyses (summary statistics presented in **appendix 8.1.8**) comparing the two forms of Aβ, revealed no species-specific difference in their effect on pericyte contraction. This is surprising as Aβ_{1-42} is much more hydrophobic and displays a greater tendency to aggregate (375, 376) and is more abundant in the brain parenchyma (377) so one would expect it elicit a reduced effect compared to Aβ_{1-40}. Aβ_{1-40} is less hydrophobic and typically collects in blood vessels (378), as is seen in arteriosclerosis and CAA (62, 379). It is likely to be the most common isoform to which vascular pericytes are exposed and so it is interesting that it does not elicit a significantly different effect compared to Aβ_{1-42}. Numerous studies do show Aβ_{1-40} exhibits vascular effects including associated reductions in pericyte number/vessel length (380), impaired functional hyperaemia and enhanced vasoconstriction in the mouse neocortex (216, 356). It also is shown to inhibit nitric oxide signalling contributing to increased reactive oxygen species-mediated platelet activation and perturbed vasodilation (381, 382).

A study by Schulz demonstrates that the assembly of Aβ_{1-40} has opposite effects on cell proliferation: monomeric Aβ_{1-40} induced proliferation whereas fibrillar Aβ_{1-40} reduced proliferation of NG2+ve human pericytes (286). Aggregation states were not compared in the present study, which is a major limitation of the work, and something that needs to be addressed in future studies.

In this chapter, I have shown that exposure to high concentrations of Aβ impair EDN1-mediated pericyte contraction. The deleterious impact of Aβ on pericyte responsiveness – impairing the contractile response – is likely to interfere with the rapid adaptation of capillary blood flow to changes in neuronal metabolic demand, and suggests a mechanism whereby altered Aβ may contribute to neurovascular uncoupling and cerebral hypoperfusion in AD. It remains to be determined the extent to which the aggregation states of Aβ (monomeric, oligomeric and fibrillar) influences pericyte contractile function. Fibrillar forms of Aβ_{1-40} induced cell death,
caspase activity and reduced proliferation of human brain vascular pericytes but these effects were not found with oligomeric Aβ peptide(380). This is of particular interest since oligomeric and not fibrillar Aβ1-42 is regarded as the more toxic form of Aβ in the AD brain(383). Instead, the authors found that monomeric preparations of Aβ1-40 were associated with a significant increase in pericyte number compared to untreated cells(380), consistent with other studies demonstrating similar mitogenic properties in other cell types(384) as well as other beneficial effects of Aβ1-40 such as preventing cell membrane degradation(385). The impact of Aβ on pericyte responses to vasodilators such as NO, adenosine and PGE2 also merits further study.

4.5.2 Summary of key points

Aβ peptides alter EDN1 mediated pericycle contraction: Aβ peptides at supra-physiological levels impair EDN1-mediated pericycle contraction, possibly due to toxic effects which is concentration but not species dependent.

How Aβ exerts its modulatory effects on the EDN1-mediated constriction of capillaries and subsequent reduced CBF is still not fully understood. EDNRs are likely to play a critical regulatory role in EDN-1 mediated pericycle contraction. The effect of Aβ peptides on EDN1 receptor expression in pericytes in vitro is explored in Chapter 5 and the distribution and expression of EDN1 receptors is characterised in human post-mortem brain tissue in AD in Chapter 6.
Chapter 5  Investigating the effects of Aβ on endothelin receptor expression

5.1  Background

In the previous chapter, I reported changes in EDN1-mediated contractile responses of fHBVP following Aβ exposure (high Aβ concentrations impeding contraction). How Aβ exerts these effects is unclear but EDNRs could play a role.

EDNRA and EDNRB are two GPCRs consisting of seven hydrophobic transmembrane domains with an extracellular N terminus and intracellular C terminus. Both endothelin receptors are coupled to multiple but distinct second messengers. EDNRA activation stimulates cAMP formation, whereas activation of EDNRB inhibits cAMP formation while increasing phosphoinositide turnover and mitogen-activated protein kinase activation. EDN1 has been shown to induce adenylate cyclase activity in EDNRA-expressing cells, indicating a direct link between EDNRA and adenylate cyclase signalling, important for smooth muscle contraction in the heart and lung.

In the cardiovascular system, EDNRAs are predominantly found on VSCMs and produce vasoconstriction via increased release of intracellular Ca²⁺ following receptor activation. Conversely EDNRBs are located on vascular endothelial cells where they produce vasodilation via nitric oxide synthase activation through calcium-calmodulin and tyrosine kinase-dependent mechanisms.

It was in 1992 that these receptors were first demonstrated within the brain, predominantly in the cerebrum, cerebellum, and brain stem. In vivo work illustrated regional localisation of EDNRA in the thalamus, hypothalamus and mid- and hind-brain whilst EDNRB mRNA labelling was observed mainly in the Purkinje cells of the cerebellum, choroidal epithelial cells, glial cells, and ependymal cells of the ventricles. EDNRA was thought to be predominantly expressed in VSMC within the brain while EDNRB is present in a wider variety of cell types, predominantly in vascular endothelial cells and glial cells. Pericytes isolated from bovine retinal and brain capillaries have been shown to express EDNRA and EDNRB and play important roles in mediating pericyte contraction in response to EDN1 as shown in vitro and in rat cortical brain tissue.

In a previous in vivo study of the effects of Aβ on endothelin receptor expression, EDNRA increased in response to Aβ₁₋₄₀ in the brain. Rats treated with Aβ₁₋₄₀ showed marked increased
EDNRA in the cerebral cortex, hippocampus, and brain stem by 72%, 85%, and 90%, respectively (369). This potentially highlights an adaptive response in that EDNRA is increased because of Aβ-mediated impairment of pericyte contraction. Other regions were not affected, suggesting these changes are specific to brain regions most susceptible to Aβ-mediated deposition. Increased EDNRA expression was significantly attenuated in rats treated with the selective EDNRA antagonist (BQ123) and non-specific EDNRA/EDNRB antagonist (TAK-044) indicating a potentially reversible relationship. No effect on EDNRB expression in these same brain regions was observed (369), indicating that the Aβ-mediated changes are primarily of a vascular nature.

Together, these findings suggest that EDNRA and EDNRB have important roles in regulating CBF and that Aβ-mediated dysregulation of EDNRA and EDNRB within the cerebral vasculature is likely to contribute to reduced CBF in AD. In this chapter, the effects of Aβ on endothelin receptor expression (at the transcript and protein level) are investigated in human brain pericytes.

5.2 Hypothesis and Aims

5.2.1 Hypothesis

The hypothesis investigated in this chapter was that Aβ-induced alteration in the expression and ratio of EDNRA in human pericyte cultures underpins modulation of pericyte contraction by Aβ.

5.2.2 Aims

The aims of this study were to measure the protein levels and gene expression of endothelin receptors A and B in fHBVP and aHBVP following short (1 hour) and longer (24 hour) exposure to Aβ peptides (which I have previously shown to modulate pericyte contractility).
5.3 Methods

5.3.1 Immunofluorescent labelling of pericytes

Cells were grown on poly-L-lysine coated coverslips as detailed in section 2.2.1 overnight and were pre-incubated with Aβ peptides (0.001 – 10 µM; rPeptide, USA) for either 1 or 24 hours, prior to fixation. Expression of endothelin receptors was confirmed for each receptor by immunofluorescence using the method detailed in section 2.2.2. Primary antibodies were applied as follows; EDNRA (1:500, Abcam UK, rabbit polyclonal), EDNRB (1:1000, Abcam UK, rabbit polyclonal). Secondary antibody used was donkey anti-rabbit IgG Alexa Fluor 488 (Invitrogen, UK) for EDNRA and EDNRB.

To assess total cellular receptor expression, cells were immunolabelled as outlined in section 2.2.2. To assess membrane-bound expression only, the cells were immunolabelled without the addition of methanol for 10 minutes (to prevent intracellular access of the labelling antibody). Images from three randomly selected areas of the coverslip were acquired using a fluorescence microscope (Nikon eclipse 80i). The images were analysed using Cell Profiler imaging software to identify individual cells and calculate the mean fluorescence intensity per cell in each image and this was used to calculate the average fluorescence intensity for each condition.

5.3.2 Real-time PCR

The Cells-to-CT™ kit was used to lyse cells and collect RNA for the conversion of cDNA by reverse transcriptase. Real-time PCR was then used to measure gene expression of EDNRA and EDNRB, in extracts of fHBVP and aHBVP normalised to RPL13 and UBE2D2 reference (housekeeping) genes as detailed in see section 2.2.6.
5.4  Results

5.4.1  Acute Aβ exposure does not affect endothelin receptor expression in fHBVP

To investigate whether membrane-bound or total endothelin receptor expression was altered by short-term Aβ exposure, pericytes were treated with Aβ_{1-40} or Aβ_{1-42} at 0.001 µM, 0.01 µM, 0.1 µM, 1 µM or 10 µM 1 hour prior to fixation and subsequently immunolabelled for membrane-bound and total EDNRA and EDNRB (n = 9 independent experiments).

The level of fluorescence reflecting membrane-bound EDNRA and EDNRB was measured in fHBVP. There was no significant difference between membrane-bound EDNRA or EDNRB after 1-hour exposure to either Aβ_{1-40} or Aβ_{1-42} (Figure 25). See Appendix Figure 3 for representative immunofluorescence images.
Figure 25. Change in membrane-bound endothelin receptors in fHBVP incubated with Aβ for 1 hour

(A) There was no significant difference in EDNRA labelling in cells treated Aβ₁-₄₀ compared to untreated cells (n = 9). (B) There was no significant difference in EDNRA labelling in cells treated with Aβ₁-₄₂ compared to untreated cells (n = 9). (C) There was no significant difference in EDNRB labelling in cells treated with Aβ₁-₄₀ compared to untreated cells (n = 9). (D) There was no significant difference in EDNRB labelling in cells treated with Aβ₁-₄₂ compared to untreated cells (n = 9). Kruskal-Wallis and Dunn’s multiple comparison post hoc test (A, C), RM one-way ANOVA and Dunnett’s multiple comparison post hoc test (B) and Friedman test with Dunn’s multiple comparison post hoc test (D) were used for comparisons. Data represent the mean values ± SEM.
I next assessed the level of fluorescence reflecting total fHBVP EDNRA and EDNRB (n = 9 independent experiments).

There was no significant difference in total EDNRA or EDNRB after 1-hour exposure with either Aβ₁₋₄₀ or Aβ₁₋₄₂ (Figure 26). See Appendix Figure 4 for representative immunofluorescence images.

**Figure 26. Change in total endothelin receptor expression in fHBVP incubated with Aβ for 1 hour**

(A) There was no significant difference in EDNRA labelling in cells treated Aβ₁₋₄₀ compared to untreated cells (n = 9). (B) There was no significant difference in EDNRA labelling in cells treated with Aβ₁₋₄₂ compared to untreated cells (n = 9). (C) There was no significant decrease in EDNRB labelling in cells treated with Aβ₁₋₄₀ compared to untreated cells (n = 9). (D) There was no significant difference in EDNRB labelling in cells treated with Aβ₁₋₄₂ compared to untreated cells (n = 9). RM one-way ANOVA and Dunnett’s multiple comparisons post hoc test (A), Kruskal-Wallis multiple comparisons post hoc test (B, C) and Friedman test and Dunn’s multiple comparison post hoc test (D) were used for comparisons. Data represent the mean values ± SEM.
I then looked at the ratio of membrane-bound and total EDNRA:EDNRB immunolabelling in pericytes exposed to Aβ peptides for 1 hour (n = 9). There was a significant increase in the ratio of membrane-bound EDNRA: EDNRB immunolabelling in cells treated with 0.1 µM (p = 0.0233) and 1 µM (p = 0.0268) Aβ1-40 for 1 hour compared to untreated cells (Figure 27A). There was also a significant increase in the ratio of membrane-bound EDNRA: EDNRB immunolabelling in cells treated with 0.1 µM (p = 0.0090) Aβ1-42 for 1 hour compared to untreated cells (Figure 27B). No significant differences were found in the ratio of total EDNRA: EDNRB immunolabelling in cells treated with Aβ1-40 or Aβ1-42 for 1 hour (Figure 27C-D).
Figure 27. Change in the ratio of membrane bound and total EDNRA: EDNRB receptor expression in fHBVP incubated with Aβ for 1 hour

(A) There was a significant increase in the ratio of membrane-bound EDNRA: EDNRB immunolabelling in cells treated with 0.1 µM (p = 0.0233) and 1 µM (p = 0.0268) Aβ1-40 compared to untreated cells (n = 9). (B) There was a significant increase in the ratio of membrane-bound EDNRA: EDNRB immunolabelling in cells treated with 0.1 µM (p = 0.0090) Aβ1-42 compared to untreated cells (n = 9). (C) There was no significant decrease in the ratio of total EDNRA: EDNRB immunolabelling in cells treated with Aβ1-40 compared to untreated cells (n = 9). (D) There was no significant difference in the ratio of total EDNRA: EDNRB immunolabelling in cells treated with Aβ1-42 compared to untreated cells (n = 9). Kruskal-Wallis and Dunn’s multiple comparisons post hoc test were used for all comparisons. Data represent the mean values ± SEM.
5.4.2 Long-term exposure of Aβ alters endothelin receptor expression in fHBVP

To investigate whether membrane-bound or total endothelin receptor expression was altered by chronic Aβ exposure, fHBVP were treated with Aβ1-40 and Aβ1-42 at 0.001 μM, 0.01 μM, 0.1 μM, 1 μM or 10 μM for 24 hours before fixation and were immunolabelled for EDNRA or EDNRB (n = 9 independent experiments).

The level of fluorescence reflecting membrane-bound EDNRA and EDNRB was measured in fHBVP. There was no significant difference between membrane bound EDNRA or EDNRB after 24-hour exposure to either Aβ1-40 or Aβ1-42 (Figure 28). See Appendix Figure 5 for representative immunofluorescence images.
**Figure 28. Change in membrane-bound endothelin receptors in fHBVP incubated with Aβ for 24 hours**

(A) There was no significant difference in EDNRA labelling in cells treated Aβ₁₋₄₀ compared to untreated cells (n = 9). (B) There was no significant difference in EDNRA labelling in cells treated with Aβ₁₋₄₂ compared to untreated cells (n = 9). (C) There was no significant difference in EDNRB labelling in cells treated with Aβ₁₋₄₀ compared to untreated cells (n = 9). (D) There was no significant difference in EDNRB labelling in cells treated with Aβ₁₋₄₂ compared to untreated cells (n = 9). Kruskal-Wallis and Dunn’s multiple comparison post hoc test [A, B] and RM one-way ANOVA and Dunnett’s multiple comparisons post hoc test [C, D] were used for comparisons. Data represent the mean values ± SEM.
There was a significant increase in total EDNRA in cells treated with 0.001 µM (p = 0.0216) Aβ<sub>1-40</sub> for 24 hours compared to untreated cells (Figure 29A). There was no significant difference in total EDNRA in cells treated with Aβ<sub>1-42</sub> (Figure 29B). The data show no significant difference in EDNRB in cells treated with Aβ<sub>1-40</sub> for 24 hours compared to untreated cells (Figure 29C). There was a significant decrease in EDNRB labelling in cells treated with 1 µM (p = 0.0243) and 10 µM (p = 0.0313) Aβ<sub>1-42</sub> (Figure 29D). See Appendix Figure 6 for representative immunofluorescence images.
Figure 29. Change in total endothelin receptor expression in fHBVP incubated with Aβ for 24 hours

(A) There was a significant increase in EDNRA labelling in cells treated with 0.001 µM (p = 0.00216) Aβ1-40 compared to untreated cells (n = 9). (B) There was no significant difference in EDNRA labelling in cells treated with Aβ1-42 compared to untreated cells (n = 9). (C) There was no significant difference in EDNRB labelling in cells treated with Aβ1-40 compared to untreated cells (n = 9). (D) There was a significant decrease in EDNRB labelling in cells treated with 1 µM (p = 0.0243) and 10 µM (p = 0.0313) Aβ1-42 compared to untreated cells (n = 9). RM one-way ANOVA and Dunnett’s multiple comparisons post hoc test (A, B, D) and Friedman test and Dunn’s multiple comparison post hoc test (C) were used for comparisons. Data represent the mean values ± SEM.
I then looked at the ratio of membrane-bound and total EDNRA:EDNRB immunolabelling in pericytes exposed to Aβ peptides for 24 hours (n = 9). There was a significant increase in the ratio of EDNRA:EDNRB immunolabelling in cells treated with 0.01 µM (p = 0.0030) Aβ1-40 for 24 hours compared to untreated cells (Figure 30A) but no significant difference in the ratio of membrane-bound EDNRA:EDNRB immunolabelling in cells treated with Aβ1-42 for 24 hours (Figure 30B). No significant differences were found in the ratio of total EDNRA: EDNRB immunolabelling in cells treated with Aβ1-40 and Aβ1-42 for 24 hours (Figure 30C-D).
Figure 30. Change in the ratio of membrane bound and total EDNRA: EDNRB receptor expression in fHVP incubated with Aβ for 24 hours

(A) There was a significant increase in the ratio of membrane-bound EDNRA: EDNRB immunolabelling in cells treated with 0.01 µM (p = 0.0030) Aβ1-40 compared to untreated cells (n = 9). (B) There was no significant difference in the ratio of membrane-bound EDNRA: EDNRB immunolabelling in cells treated with Aβ1-42 compared to untreated cells (n = 9). (C) There was no significant decrease in the ratio of total EDNRA: EDNRB immunolabelling in cells treated with Aβ1-40 compared to untreated cells (n = 9). (D) There was no significant difference in the ratio of total EDNRA: EDNRB immunolabelling in cells treated with Aβ1-42 compared to untreated cells (n = 9). Kruskal-Wallis and Dunn's multiple comparisons post hoc test were used for all comparisons. Data represent the mean values ± SEM.
5.4.3 Gene expression of EDNRA and EDNRB in pericytes exposed to Aβ

I next performed RT-PCR to validate the findings from the immunostaining experiments. This was to determine whether the increase in receptor expression was associated with an increase in gene expression.

5.4.3.1 Validation of reference genes in cell cultures for RT-PCR

Prior to investigating target gene expression, it was important to validate the selection of reference (housekeeping) genes RPL13 and UBE2D2. The average C_q values for each calibrator gene were calculated for fHBVP untreated (control) or treated with Aβ_{1-40} or Aβ_{1-42} (0.001 µM or 1 µM) for 24 hours (n = 15; Figure 31). Pearson’s correlation coefficient showed a significant correlation between these two reference genes (r = 0.8429; p = 0.0002). The mean C_q values of RPL13 and UBE2D2 was used to calculate 2^{\Delta\Delta C_q} for each gene target (EDNRA and EDNRB).

![Figure 31. RPL13 and UBE2D2 reference gene validation](image)

Data shows a significant correlation between RPL13 and UBE2D2 reference gene C_q values (r = 0.8429; p = 0.0002; n = 15). Pearson’s correlation coefficient was used for analysis. Data represent the mean with 95% confidence intervals.
5.4.3.2  Aβ causes increased EDNRA gene expression in fHBVP

To investigate the effects of exogenously applied Aβ peptides on endothelin receptor gene expression in fHBVP, pericytes were pre-treated with Aβ1-40 or Aβ1-42 (0.001 µM or 1 µM) for 24 hours (to repeat the same conditions that caused changes in receptor protein expression). A control comprising no Aβ pre-treatment was included (n = 3).

There was a significant increase in EDNRA in cells treated with 0.001 µM Aβ1-40 (p = 0.0459) compared to untreated cells (Figure 32A). No significant difference in EDNRA was found in cells treated with 1 µM Aβ1-40. No significant difference in EDNRA expression was observed in cells treated with wither low or high concentrations of Aβ1-42 (Figure 32B).

![Figure 32. EDNRA mRNA transcript level is increased in response to Aβ1-40](image)

(A) Significant increase in EDNRA expression in cells treated with 0.001 µM Aβ1-40 (p = 0.0459) compared to untreated cells when normalised to RPL13/UBE2D2 reference genes (n = 3). (B) There was no significant difference in EDNRA mRNA transcript level between untreated cells and those treated with Aβ1-42 (n = 3). Kruskall-Wallis and Dunn’s multiple comparisons post hoc test were used for comparisons. Data represent the geometric mean with 95% confidence intervals, showing fold change relative to the control group, normalised to RPL13/UBE2D2 mRNA expression on a log base 2 scale.

It was not possible to assess EDNRB expression as the levels of transcript in the samples were below the threshold limit for detection.
5.4.3.3 Endothelin receptor gene expression unchanged in aHBVP

In Chapter 3 I showed adult pericytes were less responsive to EDN1 than foetal pericytes were. I next wanted to determine whether this was due to altered gene expression of EDNRA and/or EDNRB. fHBVP and aHBVP were grown and EDNRA expression levels were measured by RT-PCR (n = 3).

There was no difference in EDNRA mRNA transcript level in aHBVP compared to fHBVP (Figure 33).

Figure 33. EDNRA mRNA expression is unchanged in aHBVP

There was no significant difference in EDNRA mRNA expression between fHBVP and aHBVP (n = 3). Mann-Whitney two-tailed t test was used for comparison. Data represent the geometric mean with 95% confidence intervals, showing fold change relative to the foetal group normalised to RPL13/UBE2D2 mRNA expression on a log base 2 scale.
5.5 Discussion

In this chapter, I have shown increased EDNRA protein level and gene expression in fHBVP in response to 1- and 24-hour exposure to pathophysiological levels of Aβ\textsubscript{1-40}. Higher concentrations of Aβ (1-10 μM) resulted in reduced EDNRA and EDNRB expression and may account for impaired pericyte contraction in response to EDN1 (shown in Chapter 4), possibly due to the toxic effects of supra-physiological levels of Aβ. These mechanisms could contribute to capillary constriction and cerebral hypoperfusion, and impaired neurovascular signalling, in AD.

5.5.1 Chronic exposure to Aβ\textsubscript{1-40} increases total endothelin receptor expression in fHBVP

As reported in Chapter 3, I have shown that EDN1-mediated contraction of pericytes was EDNRA-dependent. In Chapter 4, I have reported that high (potentially toxic) and pathophysiological levels of Aβ dysregulate EDN1-mediated pericyte contraction. In this chapter, I sought to determine if Aβ-induced changes in endothelin receptor expression were associated with the altered contractile response in fHBVP to EDN1 when exposed to Aβ.

I found a significant increase in the ratio of membrane-bound EDNRA:EDNRB after 1 and 24-hour and total EDNRA expression after 24-hour exposure to physiological concentrations of Aβ\textsubscript{1-40}(395). These findings agree with a previous study which revealed that Aβ\textsubscript{1-40} was associated with an increase in EDNRA expression in the cerebral cortex, hippocampus, and brain stem of rodent models of AD(369). The authors also revealed that EDNRA antagonism with BQ123 attenuated the Aβ-induced increase in receptor expression(369). Higher concentrations of Aβ (1 μM – 10 μM) reduced EDNRA and EDNRB by up to 60% but this was most likely due to cell toxicity considering the 20-50% reduction in pericyte number found in the viability assay in chapter 4 (section 4.4.4) using these same Aβ concentrations.

5.5.2 Chronic Aβ exposure increases EDNRA mRNA expression in fHBVP

In addition, I found supporting evidence of increased gene expression of EDNRA in pericytes in response to Aβ\textsubscript{1-40}. In contrast, a recent study profiling the major vascular and perivascular cell types of the human brain reported reduced EDNRA in extracellular matrix-regulating pericytes in AD along with reduced EDNRB, ACTA2 and COL4A1 expression(125). Small sample size and use of cells of different origin and methodological isolation (freshly isolated versus cultured cell lines) could account for this discrepancy.
These data are in keeping with previous studies that have demonstrated the modifying effects of Aβ on vascular function. Prolonged exposure of Aβ1-40 to rat arterioles in transgenic mice resulted in a significant effect on ATP-induced vascular response including vessel constriction by acute increases in ROS(396), indicating a direct effect of Aβ on cerebral vasculature. The effects of Aβ1-40 were reversed in rodents infused with super-oxide dismutase but Aβ1-42 did not influence CBF(361). Aβ1-40 is also shown to induce mitochondria-mediated apoptosis in vascular endothelial cells(397) leading to dysfunctional tight junctions, basement membrane thickening and vascular inflammation(398, 399). Niwa et al. directly compared the effects of soluble Aβ1-40 against Aβ1-42 in mouse cerebral circulation and observed vascular dysfunction only with Aβ1-40(199, 356). Together, these data suggest that Aβ1-40 in particular is responsible for mediating the vascular effects of Aβ peptides in AD.

Although the concentration of Aβ in the CSF in AD is estimated to be within the nanomolar range(400), the concentration in the perivascular space in AD is undefined. The concentration is likely to be higher given the disease associated impaired clearance and accumulation which has prompted studies to investigate Aβ across a wide range of concentrations. To study the effects of Aβ on pericyte degeneration, Verbeek et al. used high concentrations (25 µM) of synthetic Aβ whereas some studies have investigated smaller concentrations in the 0.1-10 µM range(252, 275). As previously discussed, (section 4.5.1), the extent to which the aggregation states of Aβ influences pericyte function remains unclear and would also prove useful for future study.

Together, the current data support the hypothesis that an increase in EDNRA, the receptor responsible for mediating contraction in response to EDN1, could contribute to the hypercontraction of pericytes and excessive constriction of capillaries in AD. Limitations of the current study include the small number of independent repeats, the limited range of Aβ peptides tested and the lack of characterisation of Aβ species after 1- and 24-hour exposure to pericytes.

The effects of the limited sample size used were evident from the large confidence intervals and high variation between independent experiments. This can be a common reflection of cell culture experiments from non-identical cell behaviours (i.e., declining cellular health) arising from different cell passages which can lead to varying results(401). Time permitting, this study would have benefited from repeat measurements to increase the sample size and thus robustness of the data.
5.5.3 Summary of key points

In summary, these data indicate that Aβ, at concentrations that closely match physiological levels in the brain, increased the gene expression and protein levels of EDNRA in brain-derived pericytes and reduced levels at higher concentrations (associated with impaired pericyte contraction). These data indicate that EDNRA expression, and the ratio of EDNDRα:EDNRβ, are altered in response to Aβ1-40 and mirror the changes in contraction measured by xCELLigence. In summary, changes in EDN1 receptor expression are likely to account for the effects of Aβ in altering EDN1-mediated pericyte contraction and contribute to cerebral hypoperfusion, and impaired neurovascular coupling, in AD.
Chapter 6  Characterising the expression of EDNRA and EDNRB in the human brain in AD

6.1 Introduction

In previous chapters, I have shown increased EDNRA expression in Aβ-stimulated pericytes as well as increased EDNRA gene expression in vitro. While cells grown in vitro are a useful biological model in helping understand complex interactions, they present a simplified snapshot of the mechanisms under investigation. Further benefit can come from studying these interactions in human tissue where the effects of a multi-cellular environment can be incorporated.

A previous study examined the distribution of both endothelin receptors in the human brain. Strong detection of EDNRA immunolabelling was observed in the CA1, CA2 and CA3 hippocampal neurons as well as in the Purkinje cells of the cerebellum and SMC of cerebral blood vessels (402). The same authors reported that the cellular distribution of EDNRB greatly exceeded that of EDNRA (found in 24 regions versus 9) and was strongest in neurons and endothelial cells on capillaries (402).

In our lab we have developed a panel of biochemical markers that have been used to assess the pathophysiology of vascular dysfunction in human brain tissue. VEGF is a potent pro-angiogenic (403) and neuroprotective (404) signalling protein that is abundant in the brain and is upregulated in response to hypoxia (405) to promote vascularisation and increase blood flow. Elevated VEGF level is a strong correlate with cerebral hypoperfusion (406) as indicated by the reduction of the ratio of MAG:PLP1, two myelin proteins which are highly sensitive and resistant (respectively) to ischemia (407). In AD, VEGF is increased in the brain (206), as well as in the CSF (408). The ratio of MAG:PLP1 was found to be decreased in the frontal cortex in AD indicating hypoperfusion of the cortex, and was associated with a reduction in PDGFRβ, a marker of pericyte number (308). Fibrinogen is a blood plasma protein and is normally confined to the blood vessels but in AD, it leaks through the defective BBB and accumulates in the brain parenchyma. Elevated levels of fibrinogen serve as a proxy marker of BBB breakdown in AD (409).

This final chapter describes my investigation into determining and mapping changes in the expression of endothelin receptors in human post-mortem FFPE brain tissue, as well as the
relationship between these receptors and biochemical markers of disease pathology and vascular function.

6.2 Hypothesis and Aims

6.2.1 Hypothesis

In this chapter, I explored the hypothesis that EDNRA gene expression and protein labelling are increased (and EDNRB is decreased) in the human AD brain compared to healthy controls and that the altered expression of endothelin receptors in AD is associated with elevated levels of disease pathology markers (Aβ and tau) and with markers of vascular dysfunction.

6.2.2 Aims

The aims of this study were to (i) determine and map changes in the gene and protein expression of endothelin receptors in human post-mortem control and AD brain tissue and (ii) investigate the relationship with biochemical markers of disease pathology (Aβ and tau) and vascular dysfunction associated with AD.
6.3 Methods

6.3.1 Immunofluorescence

Immunofluorescent labelling of FFPE brain tissue sections was carried out as detailed in section 2.2.8. Primary antibodies were applied as follows; EDNRA (1:500, Abcam UK, rabbit polyclonal), EDNRB (1:1000, Abcam UK, rabbit polyclonal), PDGFRβ (1:500, R&D Systems USA, biotinylated goat polyclonal) and Col4a1 (1:500, Sigma, mouse monoclonal). Secondary antibodies included donkey anti-rabbit IgG Alexa Fluor 488 (Invitrogen, UK) for EDNRA and EDNRB, donkey anti-mouse IgG Alexa Fluor 555 (Invitrogen, UK) for collagen IV and streptavidin Alexa Fluor 555 conjugate (Invitrogen, UK) for PDGFRβ.

Positive immunolabelling was quantified using in-house software developed for this study as detailed in section 2.2.9.

6.3.2 Real-time PCR

RT-PCR was carried out to measure gene expression of EDNRA, EDNRB in frozen human post-mortem brain tissue samples. RPL13 and UBE2D2 were used as housekeeping reference genes and PDGFRβ, COL4A1 and PECAM1 were used as cell-specific calibrator genes to which the data were normalised. The method for RT-PCR is detailed in section 2.2.10.

6.3.3 Measurement of EDNRA and EDNRB concentration by ELISA

Total EDNRA and EDNRB protein concentrations were measured by sandwich ELISA as detailed in section 2.2.11. Samples were diluted 1:200 in Sample Dilution Buffer and the protein concentrations were interpolated from a standard curve generated by serial dilution of a standard (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 ng/ml) run alongside a 2-fold dilution of sample prior to the experiment.
6.4 Results

6.4.1 Characterisation of anti-EDNRA and -EDNRB immunolabeling in FFPE human post-mortem brain tissue

I initially optimised and developed a working immunofluorescence protocol for the detection of EDNRA and EDNRB in human post-mortem brain tissue. Table 2 summarises my efforts in optimising antibodies and antigen retrieval methods.

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<th>Antigen retrieval method</th>
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<td>Thermo (PA3065)</td>
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Table 2. Immunofluorescence protocol optimisation summary

Table showing the summary details of the antigen retrieval methods used to optimise and develop a working immunofluorescence protocol for immunolabelling human post-mortem brain tissue.
It was not possible to assess EDNRA as I could not detect consistent EDNRA immunolabelling in human post-mortem FFPE brain tissue (despite testing three different antibodies and different combinations of antigen retrieval). Vascular EDNRA labelling was either absent in sections co-labelled for PDGFRβ (Figure 34A) or collagen IV (Figure 34B) or the antibody appeared to label vascular cells i.e. co-labelled with collagen-IV and PDGFRβ and non-vascular cells (probably neurons and glia) (Figure 34C-D).

Figure 34. Immunofluorescent immunolabelling of EDNRA in human post-mortem brain tissue was non-specific

Representative immunofluorescence images of the co-labelling for EDNRA and PDGFRβ and collagen IV in human post-mortem brain tissue. (A) Vascular EDNRA (green) labelling was absent in sections co-labelled for PDGFRβ (red). (B) Vascular EDNRA (green) labelling was absent in section co-labelled for collagen IV (red). (C) EDNRA (green) also appeared to be non-specific, labelling most cell types when co-stained for PDGFRβ (red) and (D) collagen IV (red). All counterstained with a nuclear marker (DAPI – blue). Magnification 60×. Scale bar represents 100 μM. Tissue section is from the parietal cortex.
6.4.2 EDNRB is highly expressed within the cerebral vasculature

FFPE human post-mortem brain tissue was double-immunolabelled to assess the localisation between EDNRB and PDGFRβ (pericyte marker) and collagen IV (basement membrane protein marker). Immunolabelling shows close association between EDNRB and both PDGFRβ (Figure 35A) and collagen IV (Figure 35B). Immunolabelling appeared to be specific to the vasculature; there was absence of any detectable labelling in non-vascular cells.

![Immunofluorescent immunolabelling of EDNRA and EDNRB](image)

**Figure 35. Immunofluorescent immunolabelling of EDNRA and EDNRB**

Representative immunofluorescence images of the co-localisation between (A) collagen IV (red) and (B) EDNRB (green) and (C) merged. Representative immunofluorescence images of the co-localisation between (D) PDGFRβ (red) and (E) EDNRB (green) and (F) merged in human post-mortem brain tissue. EDNRB immunolabelling appears to be specific to vascular cells and is absent from non-vascular cells. All counterstained with a nuclear marker (DAPI – blue). Magnification 60×. Scale bar represents 100 μM. Tissue section is from the parietal cortex.
6.4.3 **EDNRB immunolabelling is unaltered in AD**

To investigate whether EDNRB expression was altered in AD, FFPE human post-mortem control (n = 10) and AD (n = 8) brain tissue were immunolabelled for EDNRB (see Appendix Table 1 and Appendix Table 2 for summary demographics for the control and AD cohort respectively).

The mean fluorescence intensity for individually detectable positively-stained pericytes in microvessels was calculated for each image as detailed in section 2.2.9. A total of 5 images were taken per section and the average mean fluorescence was calculated and used to reflect receptor expression for each case and is presented in the figure below.

There was no significant difference in EDNRB immunolabelling between control and AD groups (Figure 36).

![Figure 36. Immunofluorescence of endothelin type B receptor in AD](image)

There was no significant difference in EDNRB immunolabelling in human post-mortem FFPE brain tissue between control and AD groups. Mean fluorescence intensity was calculated for individual positively-stained microvessels and the average across 5 images was calculated and used to reflect receptor expression. Unpaired t-test was used for comparison. Data represent the mean values ± SEM.
I was unable to produce positive immunolabeling in approximately half of the cases. I carried out repeat immunolabelling of the sections that did not work alongside a positive control and the cases again did not produce EDNRB immunolabelling, despite the positive control showing EDNRB immunolabeling. Subsequent analyses to investigate whether the lack of immunolabelling was because of post-mortem delay or age differences were carried out but no such difference was observed (see Appendix Figure 1). The reasons for the sporadic immunolabelling of different cases are currently unclear and were not investigated further.

6.4.4 EDNRA and EDNRB protein measurements

To further investigate the expression of EDN1 receptors in AD, I next measured the total protein concentrations of EDNRA and EDNRB in human post-mortem brain tissue by sandwich ELISA (n = 29; see Appendix Table 1 and Appendix Table 2 for summary demographics for the control and AD cohort respectively). EDNRA was significantly increased in AD (p = 0.0484) compared to controls (Figure 37A) but there was no difference in EDNRB protein concentration (Figure 37B). The ratio of EDNRA:EDNRB protein was also significantly higher in AD (p = 0.0017; Figure 37C).
Figure 37. EDNRA and EDNRB protein concentrations are altered in AD

(A) There was a significant increase in EDNRA protein concentration in AD compared to control (p = 0.0484; n = 29). (B) There was no significant difference in EDNRB protein concentration between control and AD (n = 30). (C) A significant increase in the ratio of EDNRA:EDNRB was found in AD compared to control brains (p = 0.0017; n = 24). Unpaired two-tailed t tests were used for comparisons. Data represent the mean ± SEM.
6.4.5 EDNRA and EDNRB gene expression in AD

In addition to protein levels, the gene expression of EDNRA and EDNRB was measured in AD across the same cases.

6.4.5.1 Validation of calibrator and reference genes in post-mortem brain tissue for RT-PCR

Prior to investigating target gene expression, it was important to validate the selection of reference (housekeeping) genes RPL13 and UBE2D2 in frozen human post-mortem brain tissue. The average Cq values for each gene were calculated (n = 35; Figure 38). Pearson’s correlation coefficient showed a significant correlation between these two reference genes (r = 0.9846; p < 0.0001). The mean Cq values of RPL13 and UBE2D2 was used to calculate $2^{\Delta\Delta Cq}$ for each gene target (EDNRA and EDNRB).

![Figure 38. RPL13 and UBE2D2 reference gene validation in post-mortem brain tissue](image)

Data shows a significant correlation between RPL13 and UBE2D2 reference gene Cq values ($r = 0.9846; p < 0.0001; n = 35$). Pearson’s correlation coefficient was used for analysis. Data represent the mean with 95% confidence intervals.
Next it was important to measure the mRNA expression levels of cell-specific calibrator genes to account for disease-related differences in cell number between samples. Three vascular genes: PDGFRβ (enriched in pericytes), PECAM1 (enriched in endothelial cells) and COL4A1 (a vascular basement membrane marker), were measured to adjust EDNRA and EDNRB gene expression (as the immunofluorescent labelling of EDNRB and most accounts in the literature suggest that EDNRA are expressed in vertebral blood vessels (319)). There were no significant differences in PDGFRβ, COL4A1 or PECAM1 mRNA expression levels between AD and control groups when normalised to RPL13/UBE2D2 mRNA expression (Figure 39; n = 35).

![Figure 39. Calibrator gene mRNA levels are unchanged in disease](image)

Data show no significant differences between (A) PDGFRβ, (B) COL4A1 and (C) PECAM1 mRNA gene expression between control and AD cohorts (n = 35). Mann-Whitney two-tailed t tests were used for comparisons. Data represent the geometric mean with 95% confidence intervals normalised to RPL13/UBE2D2 mRNA expression on a log base 2 scale.
To assess the potential impact of cerebral hypoperfusion and brain ischaemia on RNA preservation in the tissue during disease, the average \textit{RPL13/UBE2D2} \( C_q \) values were plotted against the ratio of MAG:PLP1 and VEGF, markers of tissue oxygenation and acute brain ischaemia respectively. If a reduction in \( C_q \) correlated with a reduction in MAG:PLP1 or an increase in VEGF, it would reflect damage to tissue and RNA degradation due to a lack of oxygenation and vascular damage. The data showed no significant correlation between \textit{RPL13/UBE2D2} \( C_q \) value and MAG: PLP1 (Figure 40A) and VEGF (Figure 40B).

\[ r = -0.0458 \quad (p = 0.8099) \]

\[ r = -0.2527 \quad (p = 0.1630) \]
It was also important to validate the selection of calibrator genes. The average \( C_q \) values for each calibrator gene were calculated (\( n = 35 \)). Pearson’s correlation coefficient showed a significant correlation between the newly combined \( RPL13/UBE2D2 \) reference gene and \( PDGFR\beta \) (\( r = 0.7151; p < 0.0001; \text{ Figure 41A} \)), \( COL4A1 \) (\( r = 0.7246; p < 0.0001; \text{ Figure 41B} \)) and \( PECAM1 \) (\( r = 0.9410; p < 0.0001; \text{ Figure 41C} \)). The mean \( C_q \) values of \( RPL13 \) and \( UBE2D2 \) was used to calculate \( 2^{-\Delta\Delta C_q} \) for each gene target (EDNRA and EDNRB).

![Figure 41A](image1.png)

**Figure 41A.** Reference and calibrator gene correlation

Data shows a significant correlation between combined \( RPL13/UBE2D2 \) reference gene and (A) \( PDGFR\beta \) (\( r = 0.7151; p < 0.0001; n = 35 \)) and (B) \( COL4A1 \) (\( r = 0.7246; p < 0.0001; n = 35 \)) and (C) \( PECAM1 \) (\( r = 0.9410; p < 0.0001; n = 35 \)) calibrator gene \( C_q \) values. Pearson’s correlation coefficient was used for analyses. Data represent the mean with 95% confidence intervals.
6.4.5.2 Endothelin receptor gene expression is unchanged in AD

I compared EDNRA and EDNRB expression in control and AD frozen post-mortem human brain tissue by calculating the $2^{\Delta\Delta C_q}$ for each target gene in AD and showed this as a fold change relative to the control group (n = 35).

There were no significant differences in EDNRA or EDNRB mRNA expression levels between AD and control groups when normalised to RPL13/UBE2D2 mRNA expression (Figure 42). Since there was no overall difference between controls and AD for either gene, it was unnecessary to calibrate to cell-specific markers.

![Figure 42. EDNRA and EDNRB mRNA expression are unchanged in disease](image)

(A) There was no significant difference in EDNRA mRNA expression between AD and control groups (n = 35). (B) There was no significant difference in EDNRB mRNA expression between AD and control groups. Mann-Whitney two-tailed t tests were used for comparisons. Data represent the geometric mean with 95% confidence intervals normalised to RPL13/UBE2D2 mRNA expression on a log base 2 scale.
Finally, I investigated the relationship between endothelin receptor protein concentration and gene expression (n = 26). There was a significant positive correlation between EDNRA protein and mRNA expression in AD (r = 0.5206; p = 0.0488*; Figure 43A). No significant correlation was found between EDNRB protein and mRNA expression (Figure 43B).

**Figure 43. Relationship between endothelin receptor protein concentration and gene expression**

(A) There was a significant positive correlation between EDNRA protein concentration and EDNRA mRNA expression in AD (n = 26). (B) There were no significant correlations between EDNRB protein concentration and EDNRB mRNA expression (n = 27). Spearman correlation was used for analyses. Data represent the mean with 95% confidence intervals.
6.4.6  Endothelin receptor expression is changed in relation to biochemical markers of AD and vascular dysfunction

The relationship between EDNRA and EDNRB protein concentrations and relevant biochemical measurements of markers of disease pathology (Aβ and tau) and cerebrovascular injury (MAG:PLP1, VEGF, fibrinogen, and levels of EDN1; courtesy of Dr Scott Miners) were investigated in human post-mortem brain tissue. **Table 3** gives the summary statistics for each comparison. Considering the number of comparisons carried out, a p-values of < 0.016 was considered statistically significant here.

There were significant positive correlations between Aβ plaque load and EDNRA protein level (p = 0.013) and the ratio of EDNRA: EDNRB (p = 0.009).

<table>
<thead>
<tr>
<th>Aβ plaque load (4G8) (%)</th>
<th>Soluble Aβ1-40 (pg/ml)</th>
<th>Soluble Aβ1-42 (pg/ml)</th>
<th>Tau load (%)</th>
<th>MAG:PLP1 (pg/ml)</th>
<th>VEGF (pg/ml)</th>
<th>Fibrinogen (ng/ml)</th>
<th>EDN1 (pg/ml)</th>
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</thead>
<tbody>
<tr>
<td>0.501</td>
<td>-0.011</td>
<td>0.226</td>
<td>0.347</td>
<td>-0.078</td>
<td>0.305</td>
<td>0.223</td>
<td>0.040</td>
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<tr>
<td>0.013*</td>
<td>0.940</td>
<td>0.560</td>
<td>0.277</td>
<td>0.716</td>
<td>0.130</td>
<td>0.296</td>
<td>0.843</td>
</tr>
<tr>
<td>Correlation Coefficient</td>
<td>Sig. (2-tailed)</td>
<td>N</td>
<td>24</td>
<td>26</td>
<td>25</td>
<td>13</td>
<td>24</td>
</tr>
<tr>
<td>-0.051</td>
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<td>0.241</td>
<td>0.247</td>
<td>-0.102</td>
<td>-0.023</td>
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<td>0.652</td>
<td>0.917</td>
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</tr>
<tr>
<td>Correlation Coefficient</td>
<td>Sig. (2-tailed)</td>
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<td>22</td>
<td>24</td>
<td>26</td>
<td>15</td>
<td>22</td>
</tr>
<tr>
<td>0.545</td>
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<td>-0.015</td>
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<td>0.079</td>
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<td>0.009**</td>
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<td>Sig. (2-tailed)</td>
<td>N</td>
<td>22</td>
<td>24</td>
<td>23</td>
<td>13</td>
<td>22</td>
</tr>
</tbody>
</table>

**Table 3. Relationships between endothelin receptor expression and biochemical markers of AD and vascular function**

Table shows summary statistics of the relationships between EDNRA, EDNRB and EDNRA: EDNRB with previously attained biochemical measurements in the tissue cohort. N = the sample size. Spearman’s coefficient was used for analyses. (*) signifies degree of statistical significance. Biochemical measurements courtesy of Dr Scott Miners.
6.5 Discussion

In this chapter, I have characterised the protein and gene expression of EDNRA and EDNRB in human post-mortem FFPE brain tissue. The distribution of EDNRA and EDNRB was also mapped in human brain tissue: EDNRB was primarily expressed in blood vessels and co-localised strongly with collagen-IV and overlapped with a pericyte marker (PDGFRβ). EDNRA labelling produced inconsistent results, in cases with positive labelling, EDNRA was present in blood vessels and co-labelled with collagen-IV and PDGFRβ, but also labelled non-vascular cells. The expression of both genes was unchanged in AD. In contrast there was a significant increase in EDNRA protein level measured by ELISA and EDNRA protein and mRNA expression did positively correlate in AD, whilst EDNRB remain unchanged. EDNRA level, and the ratio of EDNRA:EDNRB, positively correlated with parenchymal Aβ load in the parietal cortex.

6.5.1 EDNRA and EDNRB immunolabelling in tissue

I was unable to produce consistent immunolabelling of EDNRA in FFPE sections. In some sections, EDNRA co-localised strongly with collagen-IV and there was evidence of co-labelling in pericytes. However, non-vascular labelling was also observed in these sections (and was present in sections without vascular labelling). EDNRA expression has previously been reported to be predominantly found in pericytes and VSMC with little to no expression in glial cells(125). One study has reported evidence of EDNRA expression in neurons in the rat brain(410) and another has demonstrated a non-vascular pattern of distribution of endothelin binding sites in the brain(411); casting some doubt to the specificity of the labelling in this study.

I also optimised an immunofluorescence protocol for immunolabelling EDNRB in human post-mortem FFPE brain tissue. Resulting immunofluorescence depicts strong vascular labelling (and an absence of non-vascular labelling). I have demonstrated co-localisation or close association between EDNRB and both PDGFRβ (pericyte marker) and collagen IV (basement membrane protein marker). These findings are in-keeping with existing literature which suggests pericyte expression of EDNRB(412).

A previous study by Briyal et al. looking at the effect of EDN1 antagonism and Aβ-induced changes in endothelin receptor expression in the rat brain found that intraventricular infusion of Aβ1-40 did not change EDNRB expression and that pre-treatment with endothelin receptor antagonists similarly had no effect(369). I too found no difference in EDNRB between control and AD tissue. While Briyal’s findings support the data presented herein, it is important to note not only that one study was in vivo and the other in vitro but also that tissue of different species
was used and so physiological differences between rat and human brain make direct comparisons difficult.

6.5.2 **EDNRA protein level, and the ratio of EDNRA:EDNRB, is elevated in AD but gene expression remains unaltered**

EDNRA protein concentration, measured by ELISA, was significantly increased in AD brains compared to healthy controls, whereas EDNRB remained unchanged. Current literature supports an increase in EDNRA protein level in disease, with observed increased EDNRA immunolabelling in Aβ_{1-40} injected rodent brains(369) as well as increased EDNRA mRNA and protein level in animal(413, 414) and human(415) models of systemic and pulmonary arterial hypertension, the increases correlating with vascular resistance(416). These studies all similarly reported no effect on EDNRB expression, in keeping with my findings as reported in the present chapter. The upregulation of EDNRA in AD brain tissue, together with my in vitro observations of upregulation of EDNRA on exposure of human brain pericytes to Aβ_{1-40}, support a role for an increase in EDNRA-mediated activity of the endothelin system in AD and associated vascular dysfunction.

No significant differences were found in the levels of *EDNRA* or *EDNRB* mRNA between control and AD human post-mortem brain tissue, despite elevation of EDNRA protein level. This is not necessarily surprising as protein and transcript levels can diverge, influenced by the intracellular and extracellular environments(417, 418). Transcription of mRNA may not translate into protein products because of epigenetic or post-translational modifications targeting either mRNA or protein product for degradation(418). The major post-translational factor influencing mRNA-protein correlation is the half-life of proteins(419), influenced by several factors including protein stability, post-translational ubiquitination and localisation(420). One study showed marked increases in receptor half-life upon ligand binding. This was seen in a study conducted by Astapova and colleagues who investigated the extranuclear effects of androgen receptors on protein half-life. They found that dihydrotestosterone, a potent androgen receptor ligand, markedly extended the half-life of its own receptor protein independent of increased gene transcription and degradation(421). A similar effect could influence my findings in the present study, namely that the binding of Aβ to EDNRA(275, 369) may prolong the half-life of EDNRA, independent of altered gene transcription or targeted degradation.
The relationship between endothelin receptor levels and markers of disease pathology (Aβ and tau) and vascular dysfunction (MAG:PLP1, VEGF, fibrinogen and EDN1 levels) was investigated. EDNRA and the ratio of EDNRA:EDNRB positively correlated with parenchymal Aβ which supports previous observations of a link between EDN1 and Aβ in human tissue(206, 307, 321, 322) and is consistent with a previous study reporting increased EDNRA in Aβ1-40-injected rats(369). These results further support a link between the endothelin system and Aβ. No association was seen with tau.

The lack of correlation of endothelin receptor expression with markers of cerebral perfusion (MAG:PLP1) and acute brain ischaemia (VEGF-A) is surprising in light of previous findings from the group showing a strong relationship between EDN1 levels and cerebral hypoperfusion(206, 286). Furthermore, EDN1 has been shown to mediate pericyte contraction and constriction of capillaries in response to oligomeric Aβ(275) which is likely to contribute to cerebral hypoperfusion. Furthermore, modelling cerebral ischaemia, via middle artery occlusion, has been shown to upregulate EDNRB expression in male Wisar rats(422).

A previous study indicates an association between elevated fibrinogen levels and pericyte loss and BBB breakdown in AD(409). A role for EDN1 in BBB leakiness has been previously demonstrated in earlier studies that report disrupted BBB function and increased permeability in canine and murine brains following EDN1 administration(423-425). Studies have also reported a protective effect of endothelin receptor antagonists against EDN1-induced BBB disruption(426, 427), altogether suggesting a potential role of the endothelin system in contributing to cerebral hypoperfusion. In contrast, whilst the data presented herein indicates a positive trend between endothelin receptor expression and fibrinogen level, this was not statistically significant.

### 6.5.3 Summary of key points

EDNRA was expressed on vascular cells, including pericytes, and non-vascular cells and produced inconsistent labelling across brain sections. EDNRB labelling was localised specifically to blood vessels and co-localised with pericyte marker PDGFRβ. EDNRA protein level measured by ELISA, and ratio of EDNRA:EDNRB were elevated in AD in relation to parenchymal Aβ load but not tau, supporting earlier observations of a link between the endothelin system and Aβ in AD. Despite disease-related changes in protein, gene expression of EDNRA and EDNRB were unaltered in AD.
Chapter 7  Discussion

Recent disease modelling indicates that cerebral vascular changes, specifically reduced blood flow or cerebral hypoperfusion, neurovascular uncoupling, and BBB leakiness, contribute to cognitive decline and disease progression in the early stages of AD(191, 194). The mechanism/s that underpin cerebral hypoperfusion and neurovascular uncoupling in AD, however, remain poorly defined. Pericytes, contractile cells that are highly expressed in brain capillaries and form an essential component of the NVU, have undergone extensive investigation of their involvement in regulation of CBF and vascular dysfunction in AD(277-279, 285, 286). In this thesis, I have utilised a novel approach (xCELLigence) and describe a series of in vitro and human post-mortem studies to investigate whether EDN1-mediated contraction of pericytes in the brain serves as an underpinning mechanism leading to hyper-constriction of capillaries and the development of cerebral hypoperfusion in AD.

7.1 Characterisation of EDN1-mediated contraction of fHBVP and aHBVP

The first part of the thesis (Chapters 3-4) describes my investigation of the contractile (and proliferative) responses of human brain-derived vascular pericytes, in vitro to EDN1, and after exposure to Aβ peptides (to model a key aspect of AD), to explore the hypothesis that Aβ-induced dysregulation of EDN1-mediated pericyte contraction contributes to cerebral hypoperfusion in AD. Pericytes isolated from bovine retina and human foetal tissue were previously used to model pericyte physiology and pathology(330). Those studies illustrated that pericytes respond to multiple vasoactive peptides in the brain including contraction in response to EDN1(318). However, whether the responses of these non-human or foetal sources of pericytes reflect pericytes in the adult brain remained unclear.

Chapter 3 describes the validation and application of an electrical impedance assay, xCELLigence, to characterise the contractile, relaxation and proliferative responses of human brain-derived vascular pericytes to EDN1 in culture. Previous studies were restricted to a wrinkling assay (a silicone-gel based assay whereby contraction of embedded cells pulls the gel causing ‘wrinkles’ to appear and can be measured to reflect contractile force(428)) or the examination of pericytes isolated from bovine retina(330). The electrical impedance assay has allowed me to monitor the pericyte response to EDN1 in real time. In agreement with previous works(319, 326), I have demonstrated that EDN1 mediates pericyte contraction in foetal-derived pericytes in vitro in a classical G-protein coupled response. EDN1 mediated a dose-responsive contraction (and relaxation) of pericytes over a concentration range 0.1 nM – 1 µM.
(supporting earlier observations using similar concentrations(319, 326)). I also confirmed that the EDN1-mediated contraction was mediated by EDNRA.

A novel aspect of this work was to compare the contractile and proliferative responses of adult versus foetal-derived pericytes. EDN1-mediated contraction of both cells was EDNRA-dependent. However, fHBVP were more sensitive than aHBVP to EDN1. Adult pericytes required a 100-fold greater concentration of EDN1 to elicit a contractile response and the rate of relaxation was much lower with some cells remaining partially contracted. Adult-derived pericytes expressed lower levels of EDNRA and α-SMA, compared to foetal cells, which may account for the reduced responsiveness of adult cells. Reduced responsiveness and incomplete recovery (relaxation) of cells to resting state have detrimental consequences. Sustained pericyte contraction may further amplify ischaemia and reperfusion-induced injury, limit substrate delivery to the tissue and generate oxygen and nitrogen radicals which could further perturb pericyte function.

Non-identical cell behaviours as well as altered phenotypical and functional differences between ‘younger’ and ‘aged’ cells, as in the example of receptor desensitisation(349) (see section 3.5.2), may also contribute to the differences. Numerous studies indicate that during ageing, pericyte dysfunction is associated with mitochondrial, migratory and phenotypic changes(429-431), with ultrastructural studies reporting changes in mitochondrial size, and other studies showing changes in smooth muscle and lipofuscin expression(432, 433). One study even reports a significant increase in smooth muscle-γ actin fibres(348) which are associated with increased cortical stiffness. It would be interesting to further explore the different responses between foetal and adult-derived pericytes, paying particular attention to their phenotypic and functional differences. Overall, however, my findings suggest that the reduced sensitivity of adult cells to EDN1, due to a reduction in EDNRA and α-SMA expression, may contribute to dysregulated blood flow, i.e., neurovascular uncoupling, in aged individuals.

The proliferation of fHBVP in response to EDN1 was not seen in aHBVP which could be accounted for by the reasons discussed above, as well as the general decline in the proliferative capacity of cells with normal ageing. Whilst no formal investigation into the differences between proliferative capacities of these cells was carried out, it was evident from sub-culturing the aHBVP throughout the project that they proliferated to a much lesser degree, with noticeably smaller increases in total number of cells between each passage. Pericyte proliferation of fHBVP was not inhibited by EDNRA antagonism suggesting an involvement of different intracellular signalling pathways and/ or receptor activation, perhaps involving PDGFB/PDGFRβ signalling as
EDN1 has been shown to regulate proliferation via this signalling pathway in SMCs (344-347). This study also highlights a novel marked reduction in the relaxation response in the adult pericyte population. This suggests that capillary pericytes may remain partially contracted, as reported in models of ischaemia (304) offering a candidate mechanism for the augmented vasoconstriction in support of the hypothesis that capillary constriction leads to a cerebral hypoperfusion in AD.

It is worth noting that in these studies I did not check the quality or batch-to-batch consistency of the cells, all obtained commercially. The foetal cells from ScienCell have been used extensively in studies (434-437). They express pericyte markers including PDGFRβ (436) and α-SMA (which I confirmed in this study). There are concerns about the origin of these cells. Some researchers within the field suggest that their morphology resembles perivascular fibroblasts. Personal communication (Professor Andy Randall) also suggests that their transcriptomic profile does not resemble pericytes in human brain tissue. ScienCell do not disclose any information about the way in which these pericytes are obtained. I did not use cells beyond passage > 8 as it was clear that they underwent transformation and slowed in their proliferation after long-term passage. The reduced proliferative capacity of adult pericytes may also be due to differences in APOE genotype. One study has reported accelerated pericyte degeneration in AD APOE-ε4 carriers compared to ε3 and ε2 (438). APOE genotype, specifically APOE-ε4, has also been shown to negatively affect pericyte mobility and their overall ability in maintaining BBB integrity (439). Investigating the proliferative response of pericytes expressing APOE-ε4 and in aged brains would be of interest.

The characterisation of EDN1 and EDNRA in the contraction of HBVP provides an opportunity for therapeutic intervention and development. A small study demonstrated cognitive improvement in pulmonary hypertension patients receiving EDNRA blockers (440). Results from the SERAPHIN (441, 442) and AMBITION (443) trials further support significant reductions in primary end points and patient survival (444) with EDNRA blockers in patients with pulmonary arterial hypertension. A recent study looking at the effects of Zibotentan, a selective EDNRA antagonist, on cerebral hypoperfusion demonstrated its administration ameliorated Aβ1-40-induced hypertension in transgenic rats (445) suggesting EDNRA antagonism could prove useful in managing reduced CBF. There are currently three EDNRA antagonists approved for clinical use in the USA to treat pulmonary arterial hypertension: ambrisentan, bosentan and macitentan (442). The potential of these treatments in AD is unknown; however, the central role
of EDNRA in mediating vasoconstriction (in response to oligomeric Aβ(275)) provides strong evidence in favour of EDNRA’s candidacy as a treatment target to reverse reduced CBF in AD.

7.2 Aβ peptide-mediated dysregulation of pericycle contraction

Chapter 4 describes experiments aimed to determine if EDN1-mediated contraction of pericycle cultures was dysregulated in the presence of exogenously applied Aβ (to mimic aspects of disease conditions in the AD brain).

A recent study by Nortley et al. demonstrated that capillaries were abnormally constricted near Aβ plaques in human biopsy brain tissue(275). Mechanistic studies in rat cortical brain slices, revealed that oligomeric Aβ induced oxidative stress resulting in EDN1-mediated pericycle contraction and capillary constriction (in close proximity to pericycle cell soma). By utilising xCELLigence, the data presented in chapter 4 demonstrates inhibition of the contractile response upon chronic (24 hour) pre-exposure of fHBVP to Aβ concentrations like those tested by Nortley (0.1 µM).

The ability of Aβ to alter EDN1-mediated pericycle contraction is likely to impact neurovascular coupling and contribute to hyper-constriction of capillaries resulting in cerebral hypoperfusion in AD. It is important to note that the effects of high Aβ concentrations could be due to Aβ-induced toxicity. It was not possible to assess the effects of Aβ on the adult pericycle population due to deteriorating viability of the adult pericytes in culture. This study would benefit from further optimisation of this technique to re-assess the effects of long-term exposure on adult pericycle populations as well as the varied effects of the conformation of Aβ used. However, it would be expected that monomeric preparations of Aβ form fibrillar forms after 24 hours in culture and it is therefore difficult to determine from its initial physical state, the conformation of Aβ that is responsible for mediating longer term effects on pericycle contractility.

How Aβ exerts modulatory effects on EDN1-mediated contraction formed the basis for the next part of the project. EDN1 binding to EDNRA activates downstream Gq signalling cascade via phospholipase C resulting in the generation if IP3 to stimulate intracellular Ca2+ release and activate plasma membrane Ca2+ channels and stimulate influx from the extracellular space(446). The rise in cytosolic Ca2+ leads to the reorganization of actin filaments(336). In Chapter 5, I investigated whether Aβ influenced endothelin receptor expression, particularly EDNRA, which could account for the Aβ-associated changes in pericycle contractility.

Short-term (1-hour) exposure of fHBVP to Aβ peptides significantly increased the ratio of membrane-bound EDNRA:EDNRB and longer exposure (24-hours) to Aβ1-40 significantly
increased total EDNRA (and the ratio of EDNRA:EDNRB). The increase in EDNRA, the receptor responsible for mediating contraction in response to EDN1, supports the hypothesis that Aβ-induced expression of EDNRA is likely to contribute to hyper-contraction of pericytes contributing to reduced CBF in Ada and highlights a potential homeostatic response to the decreased EDN1 sensitivity displayed by aged pericytes as shown in chapter 3. These studies do not provide mechanistic insights into how Aβ influences EDNRA expression. Aβ peptides may increase the availability of EDNRA on pericytes and therefore increase the sensitivity to external agonists allowing for more EDN1 to bind and elicit a greater contractile response. This phenomenon can be seen elsewhere such as in denervation of muscle tissue whereby upregulation of acetylcholine receptors (AChRs) is associated with increased binding of and sensitivity to α-BTX and ACh(447).

To gain some insight into the regulatory process responsible for the findings, I next explored if exposure to Aβ peptides had a similar effect on EDNRA and EDNRB gene expression. EDNRA mRNA, as for protein expression, was elevated after 24-hour exposure to Aβ1-40. The data suggest that the increase in EDNRA is in part a consequence of an Aβ-associated increase in EDNRA gene expression. Studies have previously reported significant changes in the expression of numerous pro-inflammatory genes in Aβ1-42 stimulated microglia(448). Another has shown increased gene expression of transcription and growth factors including insulin-like binding protein 3 transcription factor and insulin-like growth factor receptor in response to Aβ1-42(449). Exactly how Aβ causes an increase in gene expression is unclear. In a murine cerebral endothelial cell model, Aβ1-40 has been shown to induced hyper-methylation in the neprilysin gene promoter region, resulting in the suppression of neprilysin expression and consequently, neprilysin protein expression(450). Perhaps via epigenetic alteration, Aβ1-40 upregulates EDNRA gene expression.

7.3 Characterisation of endothelin receptor expression in AD

It has previously been reported that EDN1 levels are elevated in AD and that the extent of elevation is related to the severity of disease pathology and to markers of chronic cerebral hypoperfusion (reduced MAG:PLP1 ratio) and recent brain ischaemia (elevated VEGF)(206, 308, 408). My in vitro studies indicated that EDN1-mediated contraction of pericytes was dysregulated in the presence of Aβ peptides and that Aβ-mediated induction of the expression of EDNRA (gene and protein) may account for these observations. I therefore characterised the gene and protein expression of EDNRA and EDNRB in human post-mortem brain tissue in the
parietal cortex in AD and age-matched controls to explore the hypothesis that elevated EDNRA contributes to cerebral hypoperfusion.

Immunofluorescent labelling of human post-mortem brain tissue showed strong vascular labelling and close topographical association between EDNRB in collagen-IV labelled vessels and with the pericyte marker, PDGFRβ. These findings support those of a previous study that showed EDNRB expression in pericytes(369), and like in the present study, no difference in EDNRB between controls and AD was observed. EDNRA labelling proved to be more inconsistent and did not allow for quantitative analysis. Despite testing numerous commercially available antibodies and a wide range of epitope retrieval, only one antibody produced labelling of EDNRA (and it is not clear if this labelling was specific).

Instead, I focused on measuring the protein levels of EDNRA and EDNRB by sandwich ELISA in total brain homogenates. The data revealed that EDNRA protein levels (and the ratio of EDNRA:EDNRB) were increased in AD. Although this data does not provide information about cell specific changes, it largely supports previous studies showing elevated levels of ECE and EDN1 in human brain tissue in AD(321, 322). As for the in vitro studies, I was interested in whether an upregulation in receptor expression in respect to Aβ was reflected at the gene level. In contrast to the findings in pericytes in culture, endothelin receptor gene expression was unchanged in human brain tissue in disease. Post-translational modifications or ligand binding may account for the lack of correlation between mRNA and protein level and examining brain tissue homogenates may conceal changes within individual cell-types. One study reported agonist-induced ubiquitination of EDNRB, targeting it for degradation. However, in the case of EDNRA, it is targeted for recycling back to the plasma membrane(451). In AD, Aβ binding to and activating EDNRA(369, 445) could promote recycling of the receptor leading to dissociation of EDNRA mRNA and protein levels.

Finally, I investigated changes in EDNRA and EDNRB protein levels with markers of AD pathology (Aβ and tau) and vascular dysfunction including MAG:PLP1 ratio (chronic perfusion), VEGF (recent brain ischaemia) and fibrinogen (BBB leakiness). EDNRA level, and the ratio of EDNRA:EDNRB, were not related to the degree of cerebral hypoperfusion (MAG:PLP1) or brain ischaemia (VEGF). EDNRA and the ratio of EDNRA:EDNRB protein levels were, however, related to parenchymal Aβ plaque load. This would be expected if Aβ leads to the increase in EDNRA in AD. Previous studies show a positive association between EDN1 and BBB dysfunction. Yoshiyuki et al. have demonstrated an increase in EDN1 in rats following BBB disruption and cerebral ischaemia. Their study demonstrated amelioration of the cerebral ischaemia following infusion
of S-1039, an EDNRA antagonist (452). A different study has shown infusion of EDN1 in canine brains significantly increased BBB permeability and was similarly ameliorated by application of S-1039 (423). I found a positive trend between the ratio of EDNRA:EDNRB and fibrinogen levels, a proxy marker of BBB leakiness however, this was not statistically significant using this adjusted p-value and thus does not support previous findings. The lack of finding between BBB breakdown and endothelin system in this study is likely due to the statistical approach of analyses across multiple data sets.

7.4 Limitations of the study

Limitations of the in vitro studies include the reliance on commercially available sources of human foetal and adult pericytes. Although the foetal pericytes express pericyte markers and have been extensively used to model pericyte function in vitro (275, 326, 332), concerns regarding their origin have arisen particularly as a recent single-cell transcriptomic study (243) suggest they more closely resemble perivascular fibroblasts (personal communication with Professor Andy Randall). Furthermore, the source and method of extraction is unknown, as is the impact of cell culture on the functionality of these cells. The reliance on a single source of pericytes is also restrictive, as pericytes show heterogeneity of responsiveness in vivo, because of multiple genetic and epigenetic factors. A reductionist approach was used to investigate the specific mechanistic questions but pericytes in isolation from neurons, endothelial cells, astrocytes, and microglia cannot be examined in true physiological conditions. It is important to note that the use of different batches of cells can give rise to between-experiment variation, potentially leading to varied contractile responses as seen in the xCELLigence experiments. The normalisation of data to a baseline should account for this and ensure all data across experiments are relative to this single baseline; however, the use of two-way ANOVA analyses to test for variation between repeat experiments (summary statistics shown in appendix 8.1.8) revealed significant variation between some of the repeats. This is common when studying cells in vitro and should be considered when drawing conclusions from these type of experiments.

It is now clear that the pericytes represent a heterogenous population of cells. The properties of foetal pericytes changes during development to adulthood (431). Recent transcriptomic studies indicate that pericytes exist in at least two distinct populations (solute transport and extracellular matrix-regulating pericytes) (125) and immunohistochemical and imaging studies have identified different pericyte morphologies at different points along the vasculature,
suggesting pericytes are also functionally diverse(232, 235, 236, 453). Variation in the origin and type of pericyte may account for some of the differences between fHBVP and aHBVP responses seen in this study. Future work should involve more in-depth identification and isolation of these subtypes and characterisation of their contractile responses.

Electrical impedance measurements of contraction and proliferation provide real-time objective measurements but ultimately act as proxy indicators of contraction and proliferation [which should ideally be confirmed by another technique such as phase holographic live cell(454) or calcium imaging(455)]. Compared to phase holographic imaging, xCELLigence was easier to use. EDN1 was previously shown to mediate proliferation in a study that used xCELLigence and cell counting(326). In this study, a BRDU assay was used to confirm pericyte proliferation in response to EDN1 (Appendix Figure 2, data courtesy of Dr Scott Miners). Despite the ease of use and wide-spread application, the electrical impedance assay is low-throughput. Using only a 16-well plate reader limits the capacity to analyse subsequent samples in parallel and limits the number of conditions tested. Given the significant time needed to culture cells, only a small proportion of those cells could be analysed at any given time. This further hinders this techniques potential to accumulate large data sets and identify significant differences.

The species and conformation of Aβ used in the in vitro study are also a potential limiting factor. Studies have shown that different species and conformations of Aβ mediate different responses in the vasculature. For example, fibrillar forms of Aβ1–40 induced cell death, caspase activity and reduced proliferation of human brain vascular pericytes but these were not found with oligomeric Aβ peptide(380). The present study used monomeric preparations of Aβ1–40 and Aβ1–42. I did this to ensure a consistency in the Aβ used in the cell culture experiments. Although I do not know the effects of incubation with cells for 24 hours, I presume that most Aβ will be in an oligomeric/fibrillar conformation(106) (but this was not tested). The impact of long-term freezing of Aβ stocks may also have affected the conformation of Aβ within the stocks. There was an indication that freshly prepared Aβ had a more potent effect on EDN1 induced pericyte contraction than Aβ peptide stocks that had been frozen for several months. In human brain tissue, numerous truncated and modified forms of Aβ have been identified, as well as a range of multimeric soluble and fibrillar species. The intrinsic properties of small synthetic peptides (including their limited metabolic stability) provide challenges in replicating the wide range of Aβ species found in AD in cell culture studies and may account for some of the contradictory findings within the literature(456). In this study, I am unsure of the effects of different
conformational species and did not mitigate for the potential impact on long-term storage of Aβ peptides.

Working with post-mortem human brain tissue also has limitations. The human brain, from which post-mortem FFPE tissue samples are taken, upon removal from the host is immediately subjected to oxygen exposure, change in temperature and other external factors from which it would be otherwise protected. This could result in stress-induced structural, molecular, and genetic changes, and cause bias in subsequent studies. The agonal state and post-mortem delays often vary between donors. In previous studies, the effects of simulated post-mortem delay on proteins in frozen human brain tissue, specifically endothelin-related proteins, have been modelled and have shown that most of the proteins that I have studied are stable for up to 72 hours under post-mortem conditions(457). Working with post-mortem brain tissue also only provides a snap-shot at end of life, it produces observational data, and does readily allow for determining mechanistic insights or cause and effect. Studies in human brain slices, such as the study by Nortley, provide novel mechanistic insights although ethical restrictions and practicalities limit the use of human brain slices compared to rodent brain slices.

Lastly, the use of ELISA and RT-PCR, particularly pertaining to the human tissue studies, only provide an overall picture and not cell-specific changes in protein and gene expression. Endothelin receptors are expressed in a variety of cell types of varying levels and so these methods will not discriminate protein or gene changes on a cell-specific level. Future study would benefit from using approaches such as in-situ hybridisation, or of vessel-enriched preparations which allow for precise localisation and characterisation of any changes. Fluorescence activated cell sorting could also be used to more accurately purify specific cell populations from whole tissue homogenates to more robustly analyse gene and protein expression.

7.5 Future work

In addition to areas of further research already described, this study would benefit from additional work. Whilst the cohorts were of reasonable size, the replicability of the findings should be assessed using additional cases (and with the inclusion of cases of other dementias). Power calculations could not be performed at the start of this thesis due to a lack of previous, directly comparable data. However, the results from this thesis should allow power calculations to be made for future studies, to determine the minimum number of samples required to detect
statistical significance. Example power calculations have been performed using the pilot data in this thesis; the results are presented in appendix 8.1.8. On occasion throughout this thesis, several trends in contractile responses and protein and gene expression were observed which might have proven to be significant should a larger cohort have been studied. Further investigation into these relationships should be the first lines of inquiry in any future related study.

The identification of human brain-derived vascular pericytes still remains controversial, supported by conflicting transcriptomic and immunohistochemical studies. Future work should build on ongoing, more detailed characterisation of the phenotypic and genetic markers of cells in the cerebral vasculature, to differentiate more precisely between smooth muscle cells, perivascular fibroblasts and other contractile cell types, including pericytes at different points along the vasculature. i.e., most studies are performed in cells from rodents or human foetal-derived tissue. I show that EDN1-mediated responses in pericytes from adult cells differs markedly from foetal-derived pericytes. Efforts should be directed to replicate these studies in pericytes, either obtained from induced pluripotent stem cells, or isolated from adult brain tissue obtained from neurosurgery.

7.6 Final conclusions

In this thesis, I have characterised EDN1-mediated contraction of human brain-derived vascular pericytes and highlighted novel physiological differences in the responses of foetal-derived and adult derived cells. I have demonstrated EDNRA specificity in mediating pericyte contraction, offering a potential therapeutic target in the overactive endothelin system in AD. I have also shown Aβ-induced dysregulation of EDN1-mediated pericyte contraction which seems to be related to endothelin receptor protein and gene expression. Finally, I have mapped and characterised the expression of EDNR protein (IHC, ELISA) and genes (RT-PCR) in human post-mortem AD tissue and found increased EDNRA protein levels with higher parenchymal AD pathology (Aβ). Overall, the cell culture and ex vivo work indicate a potential role for EDN1-mediated pericyte contraction that is altered in the presence of Aβ, due to changes in receptor expression. Our data provides further evidence for a role of endothelin and endothelin receptors in mediating pericyte constriction as a major cause of vessel constriction leading to cerebral hypoperfusion in AD. This study adds to our growing understanding of the vascular contribution to dementia and AD, which has the potential to change the way that AD is diagnosed and treated in the future.
## Chapter 8 Appendices

### 8.1.1 Study cohort: Control

<table>
<thead>
<tr>
<th>BB No.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Post-mortem delay (hours)</th>
<th>Braak stage</th>
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<td>72</td>
<td>M</td>
<td>42</td>
<td>I</td>
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<td>34.7 (±17)</td>
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Appendix Table 1. Summary of demographic and neuropathological data for the control cohort

BB No. (SWDBB number). Sex M/F (Male/Female). Braak stage (0 – 6) was previously determined using the Braak and Braak method (458).
### 8.1.2 Study cohort: AD

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<th>Post-mortem delay (hours)</th>
<th>Braak stage</th>
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<td>M</td>
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<td>M</td>
<td>27</td>
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<td><strong>(±7)</strong></td>
<td><strong>32.5</strong></td>
<td><strong>(±20)</strong></td>
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Appendix Table 2. Summary of demographic and neuropathological data for the AD cohort

BB No. (SWDBB number). Sex M/F (Male/Female). Braak stage (0 – 6) was previously determined using the Braak and Braak method (458).
8.1.3 PM delay and age differences between positive and negative-EDNRB immunolabelling

(A) No difference in post-mortem delay was found between groups. (B) No difference in age was found between groups. Unpaired t test was used for comparisons. Data represent the mean ± SEM.

Appendix Figure 1. Difference in post-mortem delay and age between EDNRB immunolabelling in control and AD brain tissue

8.1.4 Validation of pericyte proliferation

A BRDU assay was carried out to confirm pericyte proliferation in response to EDN1 as assessed previously by xCELLigence. EDN1 positively correlates with increased percentage change of cells as shown in Appendix Figure 2.

fHBVP were seeded in a 96-well plate at 5,000 cells per well and left to settle overnight. Pericytes were incubated with purified recombinant EDN1 (diluted in serum free medium) for 72 hours (n = 1). A BRDU incorporation assay (Abcam, UK) was used according to the manufacturer’s instructions to assess pericyte proliferation. Data courtesy of Dr Scott Miners from the Dementia Research Group, University of Bristol (personal communication).
Appendix Figure 2. BrdU assay confirming fHBVP proliferation in response to EDN1

Non-linear regression analysis model comparing EDN1 concentration (x-axis) and percentage cell death (y-axis). Data shows curve of best fit ($r^2 = 0.9723$). Data courtesy of Dr Scott Miners, Dementia Research group, University of Bristol.
8.1.5 Membrane-bound and total EDNRA and EDNRB immunolabelling in fHBVP after Aβ exposure for 1 and 24 hours

<table>
<thead>
<tr>
<th>Concentration</th>
<th>EDNRA</th>
<th>EDNRB</th>
</tr>
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<tbody>
<tr>
<td>Aβ1-40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ1-42</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CTRL</td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>0.01 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µM</td>
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Appendix Figure 3. Representative immunofluorescence of membrane-bound EDNRA and EDNRB after 1 hour exposure to Aβ

Representative immunofluorescence images of membrane-bound EDNRA and EDNRB (green) in fHBVP exposed to Aβ1-40 and Aβ1-42 (0.001 µM – 10 µM) for 1 hour. All counterstained with a nuclear marker (DAPI – blue). Magnification 40×. Scale bar represents 100 µm.
Appendix Figure 4. Representative immunofluorescence of total EDNRA and EDNRB after 1 hour exposure to Aβ

Representative immunofluorescence images of total EDNRA and EDNRB (green) in fHBPV exposed to Aβ_1-40 and Aβ_1-42 (0.001 µM – 10 µM) for 1 hour. All counterstained with a nuclear marker (DAPI – blue). Magnification 40×. Scale bar represents 100 µm.
Appendix Figure 5. Representative immunofluorescence of membrane-bound EDNRA and EDNRB after 24 hour exposure to Aβ

Representative immunofluorescence images of membrane-bound EDNRA and EDNRB (green) in fHBVP exposed to Aβ1-40 and Aβ1-42 (0.001 µM – 10 µM) for 24 hours. All counterstained with a nuclear marker (DAPI – blue). Magnification 40×. Scale bar represents 100 µm.
Appendix Figure 6. Representative immunofluorescence of total EDNRA and EDNRB after 24 hour exposure to Aβ

Representative immunofluorescence images of total EDNRA and EDNRB (green) in rHBVP exposed to Aβ1-40 and Aβ1-42 (0.001 µM – 10 µM) for 24 hours. All counterstained with a nuclear marker (DAPI – blue). Magnification 40x. Scale bar represents 100 µm.
### Results and statistical analyses

#### Chapter 3

**xCELLigence studies (EDN1 studies)**

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</tr>
<tr>
<td>Relaxation</td>
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</tr>
<tr>
<td>Proliferation</td>
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</tr>
<tr>
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<td>BQ788 antagonism (proliferation)</td>
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| ahHBVP               |          |              |
| Contraction          | 0.0226   | Yes          |
| Relaxation           | 0.0106   | Yes          |
| Proliferation        | 0.0563   | No           |
| BQ123 antagonism (contraction) | 0.0342  | Yes          |
| BQ123 antagonism (proliferation) | 0.0635  | No           |
| BQ788 antagonism (contraction) | 0.2653  | No           |
| BQ788 antagonism (proliferation) | 0.8495  | No           |

**Endothelin receptor expression studies**

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<td>α-SMA total</td>
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#### Chapter 4

**xCELLigence studies (Aβ studies)**

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**xCELLigence studies (Aβ smaller concs)**

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<td>Aβ1-42 1-hour relaxation</td>
<td>0.9997</td>
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<td>0.5690</td>
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<td>Aβ&lt;sub&gt;1-42&lt;/sub&gt; 24 hours relaxation</td>
<td>0.9384</td>
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### Pericyte viability assay

#### Chapter 5

**Endothelin receptor expression (Aβ studies)**

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<td>Aβ&lt;sub&gt;1-40&lt;/sub&gt; 1-hour membrane bound EDNRA</td>
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<td>Aβ&lt;sub&gt;1-40&lt;/sub&gt; 1-hour total EDNRB</td>
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<td>Aβ&lt;sub&gt;1-40&lt;/sub&gt; 24 hours total EDNRA</td>
<td>0.0003</td>
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<td>0.0085</td>
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<td>&lt;0.0001</td>
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<td>Aβ&lt;sub&gt;1-42&lt;/sub&gt; 24 hours total EDNRB</td>
<td>0.0255</td>
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<tr>
<td>Ratio membrane bound EDNRA: EDNRA Aβ&lt;sub&gt;1-40&lt;/sub&gt; 1 hr</td>
<td>0.0024</td>
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<td>Ratio membrane bound EDNRA: EDNRA Aβ&lt;sub&gt;1-42&lt;/sub&gt; 1 hr</td>
<td>0.0163</td>
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<td>Ratio total EDNRA: EDNRA Aβ&lt;sub&gt;1-40&lt;/sub&gt; 1 hour</td>
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<tr>
<td>Ratio membrane bound EDNRA: EDNRA Aβ&lt;sub&gt;1-40&lt;/sub&gt; 24 hr</td>
<td>0.0039</td>
<td>Yes</td>
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<td>Ratio membrane bound EDNRA: EDNRA Aβ&lt;sub&gt;1-42&lt;/sub&gt; 24 hr</td>
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<td>Ratio total EDNRA: EDNRA Aβ&lt;sub&gt;1-40&lt;/sub&gt; 24 hours</td>
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<td>Ratio total EDNRA: EDNRA Aβ&lt;sub&gt;1-42&lt;/sub&gt; 24 hours</td>
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#### Gene expression studies

**Reference gene validation**

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<tr>
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<td>EDNRA mRNA level Aβ&lt;sub&gt;1-40&lt;/sub&gt;</td>
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<td>EDNRA mRNA level Aβ&lt;sub&gt;1-42&lt;/sub&gt;</td>
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#### Chapter 6

**Endothelin receptor gene expression studies**

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<td>PDGFRB calibrator gene levels</td>
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<tr>
<td>COL4A1 calibrator gene levels</td>
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<td>No</td>
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<tr>
<td>Protein calibrator gene levels</td>
<td>0.2331</td>
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<tr>
<td>RNA preservation (MAG: PLP1)</td>
<td>0.8099</td>
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<tr>
<td>RNA preservation (VEGF)</td>
<td>0.4942</td>
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<tr>
<td>PDGFRB calibrator gene validation</td>
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<td>Yes</td>
</tr>
<tr>
<td>COL4A1 calibrator gene validation</td>
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<td>PECAM1 calibrator gene validation</td>
<td>&lt;0.0001</td>
<td>Yes</td>
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<table>
<thead>
<tr>
<th>EDNRA expression levels</th>
<th>0.5611</th>
<th>No</th>
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<tbody>
<tr>
<td>EDNRB expression levels</td>
<td>0.7874</td>
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<tr>
<td>EDNRA protein versus EDNRA mRNA expression</td>
<td>0.181</td>
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<td>EDNRB protein versus EDNRB mRNA expression</td>
<td>0.461</td>
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**Appendix Table 3. Summary statistics of data analyses**

Table contains summary statistics of each data set through chapters 3-6. P-values of <0.05 were considered statistically significant.

**8.1.7 Relationship between endothelin receptor protein changes and confounding variables**

The relationship between EDNRA and EDNRB protein concentrations and relevant confounding variables were investigated in human post-mortem brain tissue. **Appendix Table 4** gives the summary statistics for each comparison.

There was a significant negative correlation between EDNRA protein level and age ($r = -0.5470; p = 0.0038$).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Variable</th>
<th>r value</th>
<th>p-value</th>
<th>Significant?</th>
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<tbody>
<tr>
<td>EDNRA</td>
<td>Age</td>
<td>-0.5470</td>
<td>0.0038**</td>
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<td></td>
<td>Post-mortem delay</td>
<td>0.1742</td>
<td>0.3947</td>
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<td></td>
<td>Sex</td>
<td>-</td>
<td>0.2596</td>
<td>No</td>
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<tr>
<td>EDNRB</td>
<td>Age</td>
<td>-0.3006</td>
<td>0.1277</td>
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<td></td>
<td>Post-mortem delay</td>
<td>-0.0649</td>
<td>0.7478</td>
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<td></td>
<td>Sex</td>
<td>-</td>
<td>0.4580</td>
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</table>

**Appendix Table 4. Summary of relationships between endothelin receptor protein levels and confounding variables.**

Table shows the summary statistics of correlations between endothelin receptor protein levels and confounding variables including age, post-mortem delay, and sex. There was a significant negative correlation between EDNRA and age ($p = 0.0038$).
## Two-way ANOVA summary statistics

<table>
<thead>
<tr>
<th>Data set</th>
<th>p value</th>
<th>Significant?</th>
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<td><strong>Chapter 3</strong></td>
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<td></td>
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<tr>
<td>xCELLigence studies (EDN1 studies)</td>
<td></td>
<td></td>
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<tr>
<td>Foetal vs adult contraction</td>
<td>&lt;0.0001</td>
<td>Yes</td>
</tr>
<tr>
<td>Foetal vs adult relaxation</td>
<td>&lt;0.0001</td>
<td>Yes</td>
</tr>
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<td><strong>Chapter 4</strong></td>
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<tr>
<td>xCELLigence studies (Aβ studies)</td>
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<td>Aβ&lt;sub&gt;1-40&lt;/sub&gt; vs Aβ&lt;sub&gt;1-42&lt;/sub&gt; 1-hour contraction</td>
<td>0.4269</td>
<td>No</td>
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<td>Aβ&lt;sub&gt;1-40&lt;/sub&gt; vs Aβ&lt;sub&gt;1-42&lt;/sub&gt; 1-hour relaxation</td>
<td>0.4150</td>
<td>No</td>
</tr>
<tr>
<td>Aβ&lt;sub&gt;1-40&lt;/sub&gt; vs Aβ&lt;sub&gt;1-42&lt;/sub&gt; 24-hour contraction</td>
<td>0.5793</td>
<td>No</td>
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<td>Aβ&lt;sub&gt;1-40&lt;/sub&gt; vs Aβ&lt;sub&gt;1-42&lt;/sub&gt; 24-hour relaxation</td>
<td>0.4483</td>
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<tr>
<td>xCELLigence studies (Aβ smaller concs)</td>
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<td>Aβ&lt;sub&gt;1-40&lt;/sub&gt; vs Aβ&lt;sub&gt;1-42&lt;/sub&gt; 1-hour contraction</td>
<td>0.0010</td>
<td>Yes</td>
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<td>Aβ&lt;sub&gt;1-40&lt;/sub&gt; vs Aβ&lt;sub&gt;1-42&lt;/sub&gt; 1-hour relaxation</td>
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</table>

Appendix Table 5. Summary results from two-way ANOVAs

Table shows summary statistics of two-way ANOVAs which were performed to assess differences in variation between experimental repeats. P-value of < 0.05 was considered statistically significant.

## Power calculations

Listed below are example power calculations performed based on the data presented in this thesis.

<table>
<thead>
<tr>
<th>Data set</th>
<th>α (error probability)</th>
<th>Power (1-β)</th>
<th>Effect size f</th>
<th>Population size</th>
<th>Actual power</th>
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<tr>
<td>xCELLigence studies (EDN1 studies)</td>
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<tr>
<td>Contraction (foetal)</td>
<td>0.05</td>
<td>0.95</td>
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<td>Contraction (adult)</td>
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<td>BQ123 antagonism (contraction; foetal)</td>
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<td>Aβ&lt;sub&gt;1-40&lt;/sub&gt; (1hr) contraction (foetal)</td>
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<td>0.95</td>
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<td>Aβ&lt;sub&gt;1-40&lt;/sub&gt; (24hr) contraction (foetal)</td>
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<td>Aβ&lt;sub&gt;1-40&lt;/sub&gt; 1-hour membrane bound EDNRA</td>
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**Chapter 6**

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</table>

**Appendix Table 6. G-power calculations**

Table shows summary of G-power calculations performed using data from this thesis to inform future studies doing repeated or similar work.
References


416. Lutz J, Gorenflo M, Habighorst M, Vogel M, Lange PE, Ho cher B. Endothelin-1-and endothelin-receptors in lung biopsies of patients with pulmonary hypertension due
194 to congenital heart disease. Clinical chemistry and laboratory medicine.


